Journal of NEUROCHEMISTRY

VOLUME 1, 1956/1957

L.
/57

Editorial Board

V. ENGELHARDT Moscow
A. ENGSTRÖM Stockholm
S. S. KETY Bethesda, Md.
A. POPE Waverley, Mass.
R. ROSSITER London, Ont.
J. FOLCH-PI Waverley, Mass.
E. KLENK Cologne
D. RICHTER Cardiff
M. VOGT Edinburgh
H. WAELSCH New York

Advisory Board

W. BARGMANN (Kiel)
J. BENOIT (Paris)
D. BOVET (Rome)
F. BRINK, Jr. (New York)
J. ELKES (Birmingham)
K. A. C. ELLIOTT (Montreal)
O. ERÄNKÖ (Helsinki)
V. ERSPAMER (Parma)
U. S. von EULER (Stockholm)
W. FELDBERG (London)
H. FERNANDEZ-MORAN (Caracas)
L. B. FLEXNER (Philadelphia, Pa.)
J. H. GADDUM (Edinburgh)
R. W. GERARD (Ann Arbor, Mich.)
G. W. HARRIS (London)
C. HEBB (Cambridge)
H. HYDÉN (Göteborg)
H. A. KREBS (Oxford)
O. LINDBERG (Stockholm)
R. LORENTE de NÓ (New York)
O. H. LOWRY (St. Louis, Mo.)
H. McILWAIN (London)
A. v. MURALT (Berne)
A. V. PALLADIN (Kiev)
KEITH R. PORTER (New York)
J. H. QUASTEL (Montreal)
B. SCHARRER (New York)
F. O. SCHMITT (Cambridge, Mass.)
S. G. WAELSCH (New York)
P. WEISS (New York)

Pergamon Press

New York • London *Paris • Los Angeles*

JOURNAL OF NEUROCHEMISTRY

Editors:

V. ENGELHARDT: *The Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.*
A. ENGSTRÖM: *Department of Medical Physics, Karolinska Institutet, Stockholm, Sweden.*
J. FOLCH-PI: *McLean Hospital, Waverly, Mass., U.S.A.*
S. S. KETY: *Department of Health, Education and Welfare, National Institutes of Health, Bethesda* 14, *Md., U.S.A.*
E. KLENK: *Physiologisch-Chemisches Institut der Universität, Köln, Germany.*
A. POPE: *McLean Hospital, Waverley, Mass., U.S.A.*
D. RICHTER: *Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff, England.*
R. ROSSITER: *Department of Biochemistry, University of Western Ontario, London, Canada.*
M. VOGT: *Pharmacological Laboratory, University New Buildings, Edinburgh* 8, *Scotland.*
H. WAELSCH: *New York State Psychiatric Institute, New York* 32, *N.Y., U.S.A.*

Publishing Offices: 122 *East* 55*th Street, New York* 22, *N.Y.* (*Plaza* 3-8580)

Issued in parts. Subscription per volume (*A*) $17.00 *including postage;* (*B*) *for individual subscribers certifying that the journal is for their private use,* $9.80 *including postage. Payment must be made in advance*

Subcriptions should be sent to: Pergamon Press, Inc., 122 *East* 55*th Street, New York* 22, *N.Y.*

VOL
1
1956/

LIST OF CONTENTS

NUMBER 1

J. THOMAS and H. MCILWAIN: Chloride content and metabolism of cerebral tissues in fluids low in chlorides 1

MARGARETHE HOZBAUER and MARTHE VOGT: Depression by reserpine of the noradrenaline concentration in the hypothalamus of the cat 8

R. VRBA: On the participation of the glutamic acid-glutamine system in metabolic processes in the rat brain during physical exercise 12

ROSCOE O. BRADY and ROBERT MAIN BURTON: Purification and properties of spingosine . . . 18

P. CORREALE: The occurrence and distribution of 5-hydroxytryptamine (enteramine) in the central nervous system of vertebrates 22

G. B. ANSELL and JOYCE M. NORMAN: Observations on the acetalphospholipids of brain tissue . 32

LARS SVENNERHOLM: The quantitative estimation of cerebrosides in nervous tissue 42

ELI ROBINS, DAVID E. SMITH, and KATHRYN M. EYDT: The quantitative histochemistry of the cerebral cortex—I. Architectonic distribution of ten chemical constituents in the motor and visual cortices. 54

ELI ROBINS, DAVID E. SMITH, KATHRYN M. EYDT, and RICHARD E. MCCAMAN: The quantitative histochemistry of the cerebral cortex—II. Architectonic distribution of nine enzymes in the motor and visual cortices 68

ELI ROBINS, DAVID E. SMITH, and KATHRYN M. EYDT: The quantitative histochemistry of the cerebral cortex—III. Analyses at 50-μ intervals compared with analyses by architectonic layers in the motor and visual cortices 77

JOHN M. FOSTER: Enzymatic studies of mitochondria and other constituents of lobster and squid nerve fibres 84

News and Notes 91

NUMBER 2

A. GEIGER and S. YAMASAKI: Cytidine and uridine requirement of the brain 93

F. N. LEBARON and JORDI FOLCH: The isolation from brain tissue of a trypsin-resistant protein fraction containing combined inositol, and its relation to neurokeratin 101

J. B. Brierley and H. McIlwain: Metabolic properties of cerebral tissues modified by neoplasia and by freezing 109

L. G. Abood and S. K. Abul-Haj: Histochemistry and characterization of hyauronic acid in axons of peripheral nerve 119

Doris H. Clouet and M. K. Gaitonde: The changes with age in the protein composition of the rat brain 126

Holger Hydén and Sune Larsson: The application of a scanning and computing cell analyser to neurocytological problems 134

Joseph Bernsohn, Izolina Namajuska, and Benjamin Boshes: The action of chlorpromazine and reserpine on brain enzyme systems 145

G. B. Ansell and H. Dohmen: The depression of phospholipid turnover in brain tissue by chlorpromazine 150

J. Bureš: Some metabolic aspects of Leão's spreading depression 153

J.-E. Edström: The content and the concentration of ribonucleic acid in motor anterior horn cells from the rabbit 159

P. Cohn and D. Richter: Enzymic development and maturation of the hypothalamus . . 166

Robert E. Kuhlman and Oliver H. Lowry: Quantitative histochemical changes during the development of the rat cerebral cortex 173

K. A. C. Elliott and Ernst Florey: Factor I—Inhibitory factor from brain. Assay. Conditions in brain. Simulating and antagonizing substances 181

VOL. 1 56/57

NUMBER 3

W. J. Pepler and A. G. E. Pearse: The histochemistry of the esterases of rat brain, with special reference to those of the hypothalamic nuclei 193

E. Klenk, W. Vater, und G. Bartsch: Über die Gangliosidspeicherung im Nervengewebe bei der infantilen amaurotischen Idiotie vom Typ Tay-Sachs und die bei der Konservierung des Materials in Formalin auftretenden Veränderungen 203

R. Rodnight: Cholesterol and cerebrosides in brain specimens preserved for long periods in formalin. 207

Abel Lajtha: The development of the blood-brain barrier 216

Doris H. Clouet, M. K. Gaitonde, and D. Richter: The free histadine, histamine, and arginine content of the rat brain 228

Ezio Giacobini: Quantitative determination of cholinesterase in individual sympathetic cells . . 234

P. F. Cranefield, F. Brink, and D. W. Bronk: The oxygen uptake of the peripheral nerve of the rat . 245

H. Weil-Malherbe and A. C. Drysdale: Ammonia formation in brain—III. The role of the protein amide groups and of hexosamines 250

J. B. Finean, J. N. Hawthorne, and J. D. E. Patterson: Structural and chemical differences between optic and sciatic nerve myelins 256

Huntingdon Porter and Jordi Folch: Cerebrocuprein I. A copper-containing protein isolated from brain 260

Donald F. Bogdanski, Herbert Weissbach, and Sidney Udenfriend: The distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in brain 272

Max Hamburgh and Louis B. Flexner: Biochemical and physiological differentiation during morphogenesis—XXI. Effect of hypothyroidism and hormone therapy on enzyme activities of the developing cerebral cortex of the rat 279

A. Lajtha, S. Furst, A. Gerstein, and H. Waelsch: Amino acid and protein metabolism of the brain—I. Turnover of free and protein-bound lysine in brain and other organs 289

Preliminary Note:

K. Kiyota: The proteins of the brain in patients with lowered convulsion threshold 301

NUMBER 4

MAURICE M. RAPPORT and ROBERT E. FRANZL: The structure of plasmalogens—III. The nature and significance of the aldehydogenic linkage 303

IWAO ARIOKA and HIROSHI TANIMUKAI: Histochemical studies on monoamine oxidase in the midbrain of the mouse 311

S.-O. BRATTGÅRD, J.-E. EDSTRÖM, and H. HYDÉN: The chemical changes in regenerating neurons. 316

M. K. PAASONEN, P. D. MACLEAN, and N. J. GIARMAN: 5-hydroxytryptamine (serotonin, enteramine) content of structures of the limbic system 326

A. W. BAZEMORE, K. A. C. ELLIOTT, and E. FLOREY: Isolation of factor I 334

G. PORCELLATI and R. H. S. THOMPSON: The effect of nerve section on the free amino acids of nervous tissue 340

H. MCILWAIN and OLGA GREENGARD: Excitants and depressants of the central nervous system, on isolated electrically-stimulated cerebral tissues 348

D. M. ROBERTSON: The electrophoretic separation of the soluble proteins of the brain 358

D. B. HOPE: The persistence of taurine in the brains of pyridoxine-deficient rats 364

M. WOLMAN: Histochemical study of changes occurring during the degeneration of myelin . . 370

AUTHOR INDEX

ABOOD, L. G. p. 119
ABUL-HAJ, S. K. p. 119
ANSELL, G. B. p. 32, 150
ARIOKA, IWAO p. 311
BARTSCH, G. p. 203
BAZEMORE, A. W. p. 334
BERNSOHN, JOSEPH p. 145
BOGDANSKI, DONALD F. p. 272
BOSHES, BENJAMIN p. 145
BRADY, ROSCOE O. p. 18
BRATTGÅRD, S.-O. p. 316
BRIERLEY, J. B. p. 109
BRINK, F. p. 245
BRONK, D. W. p. 245
BUREŠ, J. p. 153
BURTON, ROBERT MAIN p. 18
CLOUET, DORIS H. pp. 126, 228
COHN, P. p. 166
CORREALE, P. p. 22
CRANEFIELD, P. F. p. 245
DOHMEN, H. p. 150
DRYSDALE, A. C. p. 250
EDSTRÖM, J.-E. pp. 159, 316
ELLIOTT, K. A. C. pp. 181, 334
EYDT, KATHRYN M. pp. 54, 68, 77
FINEAN, J. B. p. 256
FLEXNER, LOUIS B. p. 279.
FLOREY, ERNST pp. 181, 334
FOLCH, JORDI pp. 101, 260
FOSTER, JOHN M. p. 84
FRANZL, ROBERT E. p. 303
FURST, S. p. 289
GAITONDE, M. K. pp. 126, 228
GEIGER, A. p. 93
GERSTEIN, A. p. 289
GIACOBINI, EZIO p. 234
GIARMAN, N. J. p. 326
GREENGARD, OLGA p. 348
HAMBURGH, MAX p. 279
HAWTHORNE, J. N. p. 256
HOPE, P. B. p. 364
HOZBAUER, MARGARETHE p. 8
HYDÉN, HOLDER pp. 134, 316
KIYOTA, K. p. 301
KLENK, E. p. 203
KUHLMAN, ROBERT E. p. 173
LAJTHA, ABEL pp. 216, 289
LARSSON, SUNE p. 134
LOWRY, OLIVER H. p. 173
LEBARON, F. N. p. 101
MACLEAN, P. D. p. 326
MCCAMAN, RICHARD E. p. 68
MCILWAIN, H. pp. 1, 109, 348
NAMAJUSKA, IZOLINA p. 145
NORMAN, JOYCE M. p. 32
PAASONEN, M. K. p. 326
PATTERSON, J. D. E. p. 256
PEARSE, A. G. E. p. 193
PEPLER, W. J. p. 193
PORCELLATI, G. p. 340
PORTER, HUNTINGDON p. 260
RAPPORT, MAURICE p. 303
RICHTER, D. pp. 166, 228
ROBERTSON, D. M. p. 358
ROBINS, ELI pp. 54, 68, 77
RODNIGHT, R. p. 207
SMITH, DAVID E. pp. 54,68,77
SVENNERHOLM, LARS p. 42
TANIMUKAI, HIROSHI p. 311
THOMAS, J. p. 1
THOMPSON, R. H. S. p. 340
UNDENFRIEND, SIDNEY p. 272
VATER, W. p. 203
VOGT, MARTHE p. 8
VRBA, R. p. 12
WAELSCH, H. p. 289
WEIL-MALHERBE, H. p. 250
WEISSBACH, HERBERT p. 272
WOLMAN, M. p. 370
YAMASAKI, S. p. 93

VOL
1
1956/

VOL.
1
56/57

JOURNAL OF NEUROCHEMISTRY

Editors:

V. ENGELHARDT: *The Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.*
A. ENGSTRÖM: *Department of Medical Physics, Karolinska Institutet, Stockholm, Sweden.*
J. FOLCH-PI: *McLean Hospital, Waverley, Mass., U.S.A.*
S. S. KETY: *Department of Health, Education and Welfare, National Institutes of Health, Bethesda* 14, *Md., U.S.A.*
E. KLENK: *Physiologisch-Chemisches Institut der Universität, Köln, Germany.*
A. POPE: *McLean Hospital, Waverley, Mass., U.S.A.*
D. RICHTER: *Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff, England.*
R. ROSSITER: *Department of Biochemistry, University of Western Ontario, London, Canada.*
M. VOGT: *Pharmacological Laboratory, University New Buildings, Edinburgh* 8, *Scotland.*
H. WAELSCH: *New York State Psychiatric Institute, New York* 32, *N.Y., U.S.A.*

Publishing Offices: 122 *East* 55*th Street, New York* 22, *N.Y.* (*Plaza* 3-8580)

Issued in parts. Subscription per volume (*A*) $17.00 *including postage;* (*B*) *for individual subscribers certifying that the journal is for their private use,* $10.00 *including postage.*
Payment must be made in advance

Subscriptions should be sent to:
Pergamon Press, Inc., 122 *East* 55*th Street, New York* 22, *N.Y.*

AUTHOR INDEX TO VOLUME I

ABOOD, L. G. and ABUL-HAJ, S. K.: Histochemistry and characterization of hyaluronic acid in axons of peripheral nerve 119
ABUL-HAJ, S. K.: See ABOOD and ABUL-HAJ 119
ANSELL, G. B. and NORMAN, JOYCE M.: Observations on the acetal-phospholipids of brain tissue 32
ANSELL, G. B. and DOHMEN, H.: The depression of phospholipid turnover in brain tissue by chlorpromazine 150
ARIOKA, IWAO and TANIMUKAI, HIROSHI: Histochemical studies on monoamine oxidase in the mid-brain of the mouse 311
BARTSCH, G.: See KLENK, VATER and BARTSCH 203
BAZEMORE, A. W., ELLIOTT, K. A. C. and FLOREY, E.: Isolation of Factor I 334
BERNSOHN, JOSEPH, NAMAJUSKA, IZOLINA and BOSHES, BENJAMIN: The action of chlorpromazine and reserpine on brain enzyme systems 145
BOGDANSKI, DONALD F., WEISSBACH, HERBERT and UDENFRIEND, SIDNEY: The distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in brain . . 272
BOSHES, BENJAMIN. See BERNSOHN, NAMAJUSKA and BOSHES 145
BRADY, ROSCOE O. and BURTON, ROBERT MAIN: Purification and properties of sphingosine . 18
BRATTGÅRD, S. O., EDSTRÖM, J.-E. and HYDÉN, H.: The chemical changes in regenerating neurons 316
BRIERLEY, J. B. and MCILWAIN, H.: Metabolic properties of cerebral tissues modified by neoplasia and by freezing 109
BRINK, F.: See CRANEFIELD, BRINK and BRONK 245
BRONK, D. W.: See CRANEFIELD, BRINK and BRONK 245
BUREŠ, J.: Some metabolic aspects of Leão's spreading depression 153
BURTON, ROBERT MAIN: See BRADY and BURTON 18
CLOUET, DORIS H. and GAITONDE, M. K.: The changes with age in the protein composition of the rat brain 126
CLOUET, DORIS H., GAITONDE, M. K. and RICHTER, D.: The free histidine, histamine, and arginine content of the rat brain 228

COHN, P. and RICHTER, D.: Enzymic development and maturation of the hypothalamus . 166
CORREALE, P.: The occurrence and distribution of 5-hydroxytryptamine (enteramine) in the central nervous system of vertebrates 22
CRANEFIELD, P. F., BRINK, F. and BRONK, D. W.: The oxygen uptake of the peripheral nerve of the rat 245
DOHMEN, H.: See ANSELL and DOHMEN 150
DRYSDALE, A. C.: See WEIL-MALHERBE and DRYSDALE 250
EDSTRÖM, J.-E.: The content and the concentration of ribonucleic acid in motor anterior horn cells from the rabbit 159
EDSTRÖM, J.-E.: See BRATTGÅRD, EDSTRÖM and HYDÉN 316
ELLIOTT, K. A. C. and FLOREY, ERNST: Factor I—Inhibitory factor from brain. Assay, conditions in brain, stimulating and antagonizing substances 181
ELLIOTT, K. A. C.: See BAZEMORE, ELLIOTT and FLOREY 334
EYDT, KATHRYN M.: See ROBINS, SMITH and EYDT 54, 77
EYDT, KATHRYN M.: See ROBINS, SMITH, EYDT and MCCAMAN 68
FINEAN, J. B., HAWETHORNE, J. N. and PATTERSON, J. D. E.: Structural and chemical differences between optic and sciatic nerve myelins 256
FLEXNER, LOUIS B.: See HAMBURGH and FLEXNER 279
FLOREY, ERNST: See ELLIOTT and FLOREY 181
FLOREY, ERNST: See BAZEMORE, ELLIOTT and FLOREY 334
FOLCH, JORDI: See LEBARON and FOLCH 101
FOLCH, J.: See PORTER and FOLCH 260
FOSTER, JOHN M.: Enzymatic studies of mitochondria and other constituents of lobster and squid nerve fibres 84
FRANZL, ROBERT E.: See RAPPORT and FRANZL 303
FURST, S.: See LAJTHA, FURST, GERSTEIN and WAELSCH 289
GAITONDE, M. K.: See CLOUET and GAITONDE 126
GAITONDE, M. K.: See CLOUET, GAITONDE and RICHTER 228
GEIGER, A. and YAMASAKI, S.: Cytidine and uridine requirement of the brain . . 93
GERSTEIN, A.: See LAJTHA, FURST, GERSTEIN and WAELSCH 289
GIACOBINI, EZIO: Quantitative determination of cholinesterase in individual sympathetic cells 234
GIARMAN, N. J.: See PAASONEN, MACLEAN and GIARMAN 326
GREENGARD, OLGA: See MCILWAIN and GREENGARD 348
HAMBURGH, MAX and FLEXNER, LOUIS B.: Biochemical and physiological differentiation during morphogenesis—XXI. Effect of hypothyroidism and hormone therapy on enzyme activities of the developing cerebral cortex of the rat 279
HAWETHORNE, J. N.: See FINEAN, HAWETHORNE and PATTERSON 256
HOPE, D. B.: The persistence of taurine in the brains of pyridoxine-deficient rats . . 364
HOLZBAUER, MARGARETHE and VOGT, MARTHE: Depression by reserpine of the noradrenaline concentration in the hypothalamus of the cat 8
HYDÉN, HOLGER and LARSSON, SUNE: The application of a scanning and computing cell analyser to neurocytological problems 134
HYDÉN, HOLGER: See BRATTGÅRD, EDSTRÖM and HYDÉN 316
KIYOTA, K.: The proteins of the brain in patients with lowered convulsive threshold . . 301
KLENK, E., VATER, W. and BARTSCH, G.: Uber die Gangliosidspeicherung im Nervengewebe bei der infantilen amaurotischen Idiotie vom TYP TAY-Sachs und die bei der Konservierung des Materials in Formalin auftrenden Veranderungen. (The storage of gangliosides in nervous tissue in Tay-Sachs disease and the changes in material preserved in formalin) . 203
KUHLMAN, ROBERT E. and LOWRY, OLIVER H.: Quantitative histochemical changes during the development of the rat cerebral cortex 173
LAJTHA, ABEL: The development of the blood-brain barrier 216
LAJTHA, ABEL, FURST, S., GERSTEIN, A. and WAELSCH, H.: Amino acid and protein metabolism of the brain—I. Turnover of free and protein bound lysine in brain and other organs . 289
LARSSON, SUNE: See HYDÉN and LARSSON 134
LEBARON, F. N. and FOLCH, JORDI: The isolation from brain tissue of a trypsin-resistant protein fraction containing combined inositol, and its relation to neurokeratin . . . 101
LOWRY, OLIVER H.: See KUHLMAN and LOWRY 173

VOL
1
1956/

MacLean, P. D.: See Paasonen, MacLean and Giarman 326
McCaman, Richard E.: See Robins, Smith, Eydt and McCaman 68
McIlwain, H. and Greengard, Olga: Excitants and depressants of the central nervous system, on isolated, electrically-stimulated cerebral tissues 348
McIlwain, H.: See Thomas and McIlwain 1
McIlwain, H.: See Brierley and McIlwain 109
Namajuska, Izolina: See Bernsohn, Namajuska and Boshes 145
Norman, Joyce M.: See Ansell and Norman. 32
Paasonen, M. K., MacLean, P. D., and Giarman, N. J.: 5-Hydroxytryptamine (serotonin, enteramine) content of structures of the limbic system 326
Patterson, J. D. E.: See Finean, Hawethorne and Patterson 256
Pearse, A. G. E.: See Pepler and Pearse 193
Pepler, W. J. and Pearse, A. G. E.: The histochemistry of the esterases of rat brain, with special reference to those of the hypothalamic nuclei 193
Porcellati, G. and Thompson, R. H. S.: The effect of nerve section on the free amino acids of nervous tissue 340
Porter, Huntington and Folch, Jordi: Cerebrocuprein I. A copper-containing protein isolated from brain 260
Rapport, Maurice and Franzl, Robert E.: The structure of plasmologens—III. The nature and significance of the aldehydogenic linkage 303
Richter, D.: See Cohn and Richter 166
Richter, D.: See Clouet, Gaitonde and Richter 228
Robertson, D. M.: The electrophoretic separation of the soluble proteins of the brain . 358
Robins, Eli, Smith, David E. and Eydt, Kathryn M.: The quantitative histochemistry of the cerebral cortex—I. Architectonic distribution of ten chemical constituents in the motor and visual cortices 54
Robins, Eli, Smith, David E., Eydt, Kathryn M. and McCaman, Richard E.: The quantitative histochemistry of the cerebral cortex—II. Architectonic distribution of nine enzymes in the motor and visual cortices 68
Robins, Eli, Smith, David E. and Eydt, Kathyrn M.: The quantitative histochemistry of the cerebral cortex—III. Analyses at 50 μ intervals compared with analyses by architectonic layers in the motor and visual cortices 77
Rodnight, R.: Cholesterol and cerebrosides in brain specimens preserved for long periods in formalin 207
Smith, David E.: See Robins, Smith and Eydt 54, 77
Smith, David E.: See Robins, Smith, Eydt and McCaman 68
Svennerholm, Lars: The quantitative estimation of cerebrosides in nervous tissue . 42
Tanimukai, Hiroshi.: See Arioka and Tanimukai 311
Thomas, J. and McIlwain, H.: Chloride content and metabolism of cerebral tissues in fluids low in chlorides 1
Thompson, R. H. S.: See Porcellati and Thompson 340
Udenfriend, Sidney: See Bogdanski, Weissbach and Udenfriend 272
Vater, W.: See Klenk, Vater and Bartsch 203
Vogt, Marthe: See Holzbauer and Vogt 8
Vrba, R.: On the participation of the glutamic acid-glutamine system in metabolic processes in the rat brain during physical exercise 12
Waelsch, H.: See Lajtha, Furst, Gerstein and Waelsch 289
Weil-Malherbe, H. and Drysdale, A. C.: Ammonia formation in brain—III. The role of the protein amide groups and of the hexosamines 250
Weissbach, Herbert: See Bogdanski, Weissbach and Udenfriend 272
Wolman, M.: Histochemical study of changes occurring during the degeneration of myelin . 370
Yamasaki, S.: See Geiger and Yamasaki 93

VOL.
1
56/57

SUBJECT INDEX TO VOLUME I

Acetalphospholipids of brain tissue, observations on 32
Age, changes with, in protein composition of rat brain 126
Aldehydrogenic linkage, nature and significance 303
Amide groups, protein, and hexosamines, role in ammonia formation in brain 250
Amino acid and protein metabolism of brain, turnover of free and protein bound lysine in brain and other organs 289
Amino acids of nervous tissue, effect of nerve section on 340
Ammonia formation in brain, role of protein amide groups and of hexosamines 250
Arginine, histidine, and histamine, free, content of rat brain 228
Axons of peripheral nerve, hyaluronic acid histochemistry and characterization in . . 119
Biochemical and physiological differentiation during morphogenesis, effect of hypothyroidism and hormone therapy on enzyme activities of developing cerebral cortex of rat . . 279
Blood-brain barrier, development of 216
Brain, amino acid and protein metabolism of, turnover of free and protein bound lysine in brain and other organs 289
Brain, ammonia formation in, role of protein amide groups and of hexosamines 250
Brain, cerebrocuprein I, a copper-containing protein isolated from 260
Brain, cholesterol and cerebrosides in specimens preserved for long periods in formalin . 207
Brain, cytidine and uridine requirement 93
Brain, distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase 272
Brain, electrophoretic separation of soluble proteins 358
Brain enzyme systems, action of chlorpromazine and reserpine on 145
Brain, inhibitory Factor I, assay, conditions in brain, stimulating and antagonizing substances 181
Brain proteins in patients with lowered convulsive threshold 301
Brain, rat, free histidine, histamine, and arginine content of 228
Brain, rat, histochemistry of esterases with special reference to those of hypothalamic nuclei 193
Brain, rat, participation of glutamic acid-glutamine system in metabolic processes during physical exercise 12
Brain, rat, protein composition, changes with age 126
Brain tissue, acetalphospholipids of, observations 32
Brain tissue, depression of phospholipid turnover by chlorpromazine 150
Brain tissue, trypsin-resistant protein fraction isolation from, contains combined inositol, relation to neurokeratin 101
Brains of pyridoxine deficient rats, persistence of taurine in 364
Central nervous system, excitants and depressants of, on isolated electrically-stimulated cerebral tissues 348
Central nervous system, of vertebrates, 5-hydroxytryptamine occurrence and distribution in 22
Cerebral cortex, quantitative histochemistry of, analyses at 50 μ intervals compared with analyses by architectonic layers in the motor and visual cortices 77
Cerebral cortex, quantitative histochemistry of, architectonic distribution of nine enzymes in motor and visual cortices 68
Cerebral cortex, quantitative histochemistry of, architectonic distribution of ten chemical constituents in motor and visual cortices 54
Cerebral cortex, rat, quantitative histochemical changes during development 173
Cerebral tissues, chloride content and metabolism in fluids low in chlorides 1
Cerebral tissues, isolated electrically-stimulated, excitants and depressants of central nervous system 348
Cerebral tissues modified by neoplasia and freezing, metabolic properties of. 109
Cerebrocuprein I, a copper-containing protein isolated from brain 260
Cerebrosides and cholesterol in brain specimens preserved for long periods in formalin . 207
Cerebrosides, in nervous tissue, quantitative estimation 42
Chemical changes in regenerating neurons 315
Chloride content and metabolism of cerebral tissues in fluids low in chlorides 1
Chlorpromazine, depression of phospholipid turnover in brain tissue by 150
Chlorpromazine and reserpine action on brain enzyme systems 145

VOL 1 1956

Cholesterol and cerebrosides in brain specimens preserved for long periods in formalin . 207
Cholinesterase in individual sympathetic cells, quantitative determination 234
Convulsive threshold, lowered, protein of brain in patients. 301
Copper-containing protein, cerebrocuprein I, isolated from brain 260
Cortex, cerebral, developing, effect of hypothyroidism and hormone therapy on enzyme activities of 279
Cortex, cerebral, quantitative histochemistry of, analyses at 50 μ intervals compared with analyses by architectonic layers in the motor and visual cortices 77
Cortex, cerebral, rat, quantitative histochemical changes during development . . . 173
Cortex, motor and visual, analyses at 50 μ intervals compared with analyses by architectonic layers 77
Cortex, motor and visual, architectonic distribution of nine enzymes 68
Cortex, motor and visual, architectonic distribution of ten chemical constituents . . 54
Cytidine and uridine requirements of brain 93
Decarboxylase, 5-hydroxytryptophan-, serotonin, and monoamine oxidase distribution in brain 272
Degeneration of myelin, histochemical study of changes occurring 370
Depressants and excitants of central nervous system, on isolated electrically-stimulated cerebral tissues 348
Developing cerebral cortex, enzyme activities of, effect of hypothyroidism and hormone therapy 279
Development of the blood-brain barrier 216
Development of cerebral cortex of rat, quantitative histochemical changes during . . 173
Development, enzymic, and maturation of hypothalamus 166
Differentiation, biochemical and physiological, during morphogenesis, effect of hypothyroidism and hormone therapy on enzyme activities of developing cerebral cortex of rat . . . 279
Electrically-stimulated, isolated, cerebral tissues, excitants and depressants of central nervous system 348
Electrophoretic separation of soluble proteins of brain 358
Enzyme activities of developing cerebral cortex of rat, effect of hypothyroidism and hormone therapy 279
Enzyme systems, brain, action of chlorpromazine and reserpine on 145
Enzymes, nine, in motor and visual cortices, architectonic distribution 68
Enzymic development and maturation of hypothalamus 166
Esterases of rat brain, histochemistry of, with special reference to those of hypothalamic nuclei 193
Excitants and depressants of central nervous system, on electrically-stimulated cerebral tissues 348
Exercise, physical, participation of glutamic acid-glutamine system in metabolic processes of rat brain during 12
Factor I, inhibitory factor from brain, assay, conditions in brain, stimulatory and antagonizing substances 181
Factor I, isolation of 334
Formalin, changes in material preserved in; storage of gangliosides in nervous tissue in Tay-Sachs disease 203
Formalin, cholesterol and cerebrosides in brain specimens preserved in 207
Freezing, and neoplasia, metabolic properties of cerebral tissues modified by 109
Gangliosides, storage in nervous tissue in Tay-Sachs disease; changes in material preserved in formalin 203
Glutamic acid-glutamine system, participation in metabolic processes in rat brain during physical exercise 12
Hexosamines, and protein amide groups, role in ammonia formation in brain 250
Histamine, histidine, and arginine, free, content of rat brain 228
Histidine, histamine, and arginine, free, content of rat brain 228
Histochemical changes, quantitative, during development of rat cerebral cortex 173
Histochemical studies of monoamine oxidase in midbrain of mouse 311
Histochemical study of changes occurring during degeneration of myelin 370
Histochemistry and characterization of hyaluronic acid in axons of peripheral nerve . . 119

VOL.
1
56/57

Histochemistry of esterases of rat brain with special reference to those of hypothalamic nuclei 193
Histochemistry, quantitative, of cerebral cortex, analyses at 50 μ intervals compared with analyses by architectonic layers in the motor and visual cortices 77
Histochemistry, quantitative, of cerebral cortex, architectonic distribution of nine enzymes in motor and visual cortices 68
Histochemistry, quantitative, of cerebral cortex, architectonic distribution of ten chemical constituents in motor and visual cortices 54
Hormone therapy, and hypothyroidism, effect on enzyme activities of developing cerebral cortex of rat 279
Hyaluronic acid, in axons of peripheral nerve, histochemistry and characterization . . 119
5-Hydroxytryptamine content of structures of limbic system 326
5-Hydroxytryptamine, occurrence and distribution in central nervous system of vertebrates . 22
5-Hydroxytryptophan decarboxylase, serotonin, and monoamine oxidase distribution in brain 272
Hypothalamic nuclei, histochemistry of esterases of, in rat brain 193
Hypothalamus, cat, depression by reserpine of noradrenaline concentration in 8
Hypothalamus, enzymic development and maturation 166
Hypothyroidism, and hormone therapy, effect on enzyme activities of developing cerebral cortex of rat 279
Inositol, combined, in trypsin-resistant protein fraction isolated from brain tissue, relation to neurokeratin 101
Isolation of Factor I 334
Leão's spreading depression, some metabolic aspects 153
Limbic system, 5-hydroxytryptamine content of strucures of 326
Lobster and squid nerve fibres, mitochondria and other constituents, enzymatic studies. . 84
Lysine, free and protein bound, turnover in brain and other organs 289
Maturation and enzymic development of hypothalamus 166
Metabolic processes in rat brain, glutamic acid-glutamine system participation during physical exercise 12
Metabolic properties of cerebral tissues modified by neoplasia and by freezing . . . 109
Metabolism, amino acid and protein, of brain, turnover of free and protein bound lysine in brain and other organs 289
Metabolism, and chloride content, of cerebral tissues in fluids low in chlorides . . . 1
Midbrain, mouse, histochemical studies on monoamine oxidase in 311
Mitochondria, and other constituents of lobster and squid nerve fibres, enzymatic studies . 84
Monoamine oxidase in midbrain of mouse, histochemical studies 311
Monoamine oxidase, serotonin, and 5-hydroxytryptophan decarboxylase distribution in brain 272
Morphogenesis, biochemical and physiological differentiation during, effect of hypothyroidism and hormone therapy on enzyme activities of developing cerebral cortex of rat . . . 279
Motor anterior horn cells from rabbit, content and concentration of ribonucleic acid . . 159
Myelin degeneration, histochemical study of changes occurring 370
Myelins, nerve, optic and sciatic, structural and chemical differences between . . . 256
Neoplasia, and freezing, metabolic properties of cerebral tissues modified by . . . 109
Nerve fibres, lobster and squid, mitochondria and other constituents, enzymatic studies. . 84
Nerve myelins, optic and sciatic, structural and chemical differences between . . . 256
Nerve, peripheral, axons of, hyaluronic histochemistry and characterization in . . . 119
Nerve, peripheral, rat, oxygen uptake of 245
Nerve section, effect on free amino acids of nervous tissue 340
Nervous tissue, cerebrosides in, quantitative estimation 42
Nervous tissue, effect of nerve section on free amino acids of 340
Nervous tissue, storage of gangliosides in Tay-Sachs disease; changes in material preserved in formalin 203
Neurocytological problems, application of scanning and computing cell analyser . . . 134
Neurokeratin, relation to trypsin-resistant protein fraction containing inositol isolated from brain tissue 101
Neurons, regenerating, chemical changes in 315
Noradrenaline concentration, depression by reserpine in hypothalamus of cat 8
Nuclei, hypothalamic, histochemistry of esterases of, in rat brain 193
Optic and sciatic nerve myeline, structural and chemical differences between . . . 256

VOL 1 1956/

Oxygen uptake of peripheral nerve of rat 245
Peripheral nerve, rat, oxygen uptake of 245
Phospholipid turnover in brain tissue, depression by chlorpromazine 150
Plasmologens, structure of, nature and significance of aldyehdogenic linkage . . . 303
Protein amide groups, and hexosamines, role in ammonia formation in brain . . . 250
Protein and amino acid metabolism of brain, turnover of free and protein bound lysine in brain and other organs 279
Protein composition of rat brain, changes with age 126
Protein, copper-containing, cerebrocuprein I, isolated from brain 260
Protein fraction, trypsin-resistant, isolation from brain tissue, contains combined inositol, relation to neurokeratin 101
Proteins of brain in patients with lowered convulsive threshold 301
Proteins, soluble, of brain, electrophoretic separation of 358
Pyridoxine-deficient rats, persistence of taurine in brains of 364
Regenerating neurons, chemical changes in 315
Reserpine and chlorpromazine action on brain enzyme systems 145
Reserpine, depression of noradrenaline concentration in hypothalamus of cat by . . . 8
Ribonucleic acid, content and concentration in motor anterior horn cells from the rabbit . 159
Scanning and computing cell analyser, application to neurocytological problems . . . 134
Sciatic and optic nerve myelins, structural and chemical differences between. 256
Serotonin content of structures of limbic system 326
Serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in distribution in brain 272
Soluble proteins of brain, electrophoretic separation of 358
Sphingosine, purification and properties 18
Spreading depression, Leão's, some metabolic aspects 153
Squid and lobster nerve fibres, mitochondria and other constituents, enzymatic studies . . 84
Sympathetic cells, quantitative determination of cholinesterase in individual cells . . 234
Taurine in brains of pyridoxine-deficient rats, persistence of 364
Tay-Sachs disease, storage of gangliosides in nervous tissue; changes in material preserved in formalin 203
Trypsin-resistant protein fraction, isolation from brain tissue, contains combined inositol, relation to neurokeratin 101
Turnover of free and protein bound lysine in brain and other organs 289
Uridine and cytidine requirement of the brain 93

VOL.
1
56/57

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 1–7. Pergamon Press Ltd., London

CHLORIDE CONTENT AND METABOLISM OF CEREBRAL TISSUES IN FLUIDS LOW IN CHLORIDES

J. THOMAS and H. MCILWAIN
Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, Maudsley Hospital, London

(*Received* 14 *November* 1955)

VOL.
1
56/57

FUNCTIONAL activity in nervous tissues involves ion movements, and several metabolic characteristics of cerebral tissues are affected by the ionic composition of the media in which they are immersed. A previous study (GORE and MCILWAIN, 1952) surveyed effects of cations on the respiration and glycolysis of separated cerebral tissues, and on their response to stimulating agents. The anions of the tissue and surrounding medium have now been examined from this point of view.

In this study, attention has been directed especially towards chloride ions, because these normally preponderate and appear to be related to the marked central effects of bromides. Also, chloride ions may be specifically involved in the transmission of inhibitory effects in the central nervous system which is disturbed by strychnine (COOMBS *et al.*, 1953).

EXPERIMENTAL

Salines—The normal chloride-containing phosphate saline was that described by RODNIGHT and MCILWAIN (1954). The corresponding chloride-free "nitrate" saline was prepared similarly, and contained: 0·127 M $NaNO_3$; 0·0051 M KNO_3; 0·00127 M KH_2PO_4 and 0·00127 M $MgSO_4$; 0·00273 M $Ca(NO_3)_2$; 0·01 M glucose, 0·01 M phosphate (Na salts) at pH 7·4 saturated with O_2.

The normal, chloride-containing bicarbonate saline was that of MCILWAIN (1951), and the corresponding nitrate saline contained: 0·119 M $NaNO_3$; 0·0048 M KNO_3; 0·0012 M KH_2PO_4; 0·0012 M $MgSO_4$; 0·0025 M $Ca(NO_3)_2$; 0·0101 M glucose; 0·025 M $NaHCO_3$ gassed with 95% O_2—5% CO_2. In a few experiments only, salines with acetate as main anion were employed; these contained: phosphate saline, 0·127 M sodium acetate; 0·0051 M potassium acetate; 0·00127 M KH_2PO_4 and 0·00127 M $MgSO_4$; 0·00273 M calcium acetate; 0·01 M glucose; 0·01 M phosphate (Na salts) at pH 7·4 gassed with O_2. Bicarbonate medium contained: 0·119 M sodium acetate; 0·0048 M potassium acetate; 0·0012 M KH_2PO_4 and 0·0012 M $MgSO_4$; 0·00254 M calcium acetate; 0·0101 M glucose and 0·025 M $NaHCO_3$, and was equilibrated with 5% CO_2 in O_2. All stock acetate-salt solutions were adjusted to pH 7·4 with acetic acid before use.

Tissue and procedure—Slices of guinea-pig cerebral cortex were cut 0·35 mm in thickness (MCILWAIN, 1951) and usually floated to a shallow dish of the saline to be used in the later part of an experiment. Here they were trimmed to fit silver-grid electrodes *H* used in conical manometric vessels *A* (AYRES and MCILWAIN, 1953). When six slices had been cut (three successively from the outer to the inner part of each hemisphere), they were drained from saline, weighed on a torsion balance, fitted to their electrodes, and placed in the corresponding vessel containing 3·5 ml of the experimental saline. Electrical pulses were supplied and measured by the apparatus of AYRES and MCILWAIN (1953); unless otherwise described, alternating condenser pulses of exponential voltage-time relationship, time-constant 0·4 msec, peak potential 10 V, were applied at 100/sec. Substances added during an experiment were initially in a side-arm. Manometric measurements were normally recorded each 5 min.

Glycolysis—This was followed whenever possible by determining lactic acid according to BARKER and SUMMERSON's (1941) method. Nitrates and bromides, however, interfere with the determination,

1

and in their presence an approximate measure of glycolysis was obtained by following gas changes manometrically in bicarbonate salines in equilibrium with O_2—5% CO_2 (for an appraisal of such data, see MCILWAIN and GRINYER, 1950): this measures total acid formation and assumes a respiratory quotient of unity.

Chloride estimation—For analysis of the tissue, it was removed promptly at the end of an experiment, rinsed quickly (1 sec) in 20 ml of fresh chloride-free saline otherwise similar to that in which it had been incubated, and one of the following methods applied.

1. Titration with mercuric nitrate (SCHALES and SCHALES, 1941). The rinsed slice was added to 2·9 ml of 0·021 M sodium tungstate in 0·046 M H_2SO_4 in a test-tube homogenizer, ground, and centrifuged after 30 min; aliquots of the supernatant were titrated with 0·0168 N $Hg(NO_3)_2$ in a microburette, with diphenylcarbazone as indicator.

2. Silver titration (SUNDERMAN and WILLIAMS, 1933). Rinsed slices were digested over a steam-bath with 15 ml N KOH for 90 min, cooled, neutralized with 6 N HNO_3 and 1 ml 0·020 M $AgNO_3$, and 5 ml conc. HNO_3 added. Digestion was continued for a further 3 h. After filtering, the excess silver in the filtrate was titrated against 0·02 N NH_4CNS, using ferric alum as indicator.

3. Microdiffusion (CONWAY, 1947). Washed slices were treated with tungstic acid, as described above, and the supernatant was transferred to the outer chamber of a Conway unit containing $KMnO_4$. The central chamber contained 0·1 ml M KI; reaction was started by adding 0·2 ml 75% (*v*/*v*) H_2SO_4 to the outer chamber, and after 2 h the contents of the inner one were titrated with 0·005 N thiosulphate.

RESULTS

The Chloride of the Separated Tissue

Chloride content in the cortex of the guinea-pig cerebral hemisphere was found greater in the outer than in inner layers (Table 1). This trend, and the chloride levels, are similar to those found in part of the hippocampus of the rabbit by LOWRY *et al.* (1954). More glycogen (LEBARON, 1955) and greater glycolysis (MCILWAIN and TRESIZE, 1956) have also been found in the outer layers of guinea-pig cerebral hemispheres.

The greater part of this chloride was readily lost when the tissue was left for some minutes in chloride-free salines. In such salines, previous observations with intact animals (RAPOPORT and WEST, 1950) led us to replace chlorides by nitrates; observations with acetates were also made. As preparation of the tissue for metabolic experiments ordinarily involved a sojourn in salines, the tissue at the beginning of incubation in chloride-free salines was found (Table 1) to contain only some 2 μmoles of chloride/g, or one-twentieth of its normal level. Subsequent incubation did not lead to loss of the small quantity of chloride remaining. Determination of the chloride by the two very different methods quoted (SUNDERMAN and WILLIAMS, 1933; CONWAY, 1947) assured its identity (see also WILLIAMS *et al.*, 1954); the less specific method (SCHALES and SCHALES, 1941) involving titration with $Hg(NO_3)_2$ was found to give erroneously high values for the chloride of the tissue from chloride-free media.

Respiration in Chloride-free Media

Respiration of cerebral tissues for up to 2 h was unaffected by substitution of nitrate for chloride in glucose-phosphate salines (Table 2). Respiration stimulated by various agents, especially electrically, has proved more sensitive to changes in medium than respiration as ordinarily observed (MCILWAIN, 1953; LEWIS and MCILWAIN, 1954). Accordingly, three agents stimulating the respiration of cerebral tissues were examined in chloride-free salines: ammonium salts, additional potassium salts, and electrical pulses. All were found to have their normal effect. In addition to the respiratory rates, quoted in Table 2, the promptness of action of the agents,

TABLE 1.—CHLORIDE OF GUINEA-PIG CEREBRAL CORTEX

Several cortical areas were included in the slices, which did not show simple and clear-cut histological differences. The outer slice contained somewhat more nonmyelinated axons and their branches than the others, the second rather more cell bodies, and the third more myelinated fibres.

Position of slice	Chloride (μmoles/g moist tissue)		
	Immediately after cutting	After floating in saline, before incubation	After 40 min incubation
First (outer)	45·4[1]	2·7 ± 0·2 (4)[2]	2·4 ± 0·1 (3)[2]
Second	37·5[1]	2·0 ± 0·6 (4)[3]	1·5 ± 0·3 (4)[3]
Third	34·8[1]		

Slices were floated at room temperature (18–20°) or incubated at 37° in the nitrate-phosphate-glucose saline. Chloride determinations:

[1] average of duplicates which agreed within 2 μmoles/g (SCHALES and SCHALES, 1941);

[2] (Sunderman and Williams, 1933) and [3] (CONWAY, 1947) mean and standard deviation of the number of observations quoted in parentheses, all determinations were made with approx. 0·3 g of tissue combined from different positions in the cerebral cortex.

TABLE 2.—CHLORIDE AND AGENTS STIMULATING RESPIRATION

Applied agent	Period (min)	Respiration (μmoles O_2/g/h)	
		Normal medium	Chloride-free
None	0–90	65 ± 4 (6)	63 ± 3·7 (6)
None	0–120	65 ± 3 (6)	63 ± 4·5 (6)
None	0–60	60 ± 3 (6)	64 ± 4 (6)
30 mM KNO_3	60–120	115 ± 10 (6)	111 ± 7 (6)
None	0–40	61 ± 1 (4)	64 ± 3 (4)
Electrical pulses	40–80	115 ± 9 (4)	133 ± 12 (4)
None	0–75	62 ± 4 (4)	66 ± 3 (4)
Electrical pulses	75–115	117 ± 16 (4)	115 ± 11 (4)
None	0–35	65 (2)	66 (2)
1 mM NH_4NO_3	35–115	69 (2)	71 (2)
None	0–35	63 ± 3 (4)	64 ± 4 (4)
1 mM NH_4NO_3	35–75	73 ± 5 (4)	70 ± 5 (4)
1 mM NH_4NO_3 and electrical pulses	75–115	115 ± 7 (4)	114 ± 10 (4)

Respiration was followed manometrically, and the mean values quoted are followed by their standard deviation and number of observations. The periods of the second column are timed from the placing of the vessels at 37°.

VOL. 1 56/57

and the maintenance of their effect during the experiments, were independent of the presence of chloride.

Glycolysis in Chloride-free Media

Glycolysis also proved independent of chloride (Table 3) both in the presence and absence of stimulating agents. The course of glycolysis, as judged by gas evolution, as well as its extent, was the same in the nitrate as in the chloride medium.

TABLE 3.—GLYCOLYSIS WITH VARYING CHLORIDE AND OTHER AGENTS

Applied agents		Period (min)	Glycolysis (μmoles CO_2/g during period quoted in col. 2)	
Bromide or strychnine (S) (mM)	Others		Normal medium	Chloride-free
None	None	0–90	14	14
Br, 50	None	0–90	8	12
None	None	0–45	10	8
None	30 mM KNO_3	45–90	30	27
Br, 50	None	0–45	9	8
Br, 50	30 mM KNO_3	45–90	28	25
None	None	0–45	16	18
None	Electrical pulses	45–90	105	93
Br, 50	None	0–45	27	29
Br, 50	Electrical pulses	45–90	126	120
S, 10^{-4}	None	0–45	25	26
S, 10^{-4}	Electrical pulses	45–90	35	30
None	1 mM NH_4NO_3	0–40	54	56
None	1 mM NH_4NO_3 and electrical pulses	40–80	93	95

Glycolysis was followed manometrically, and results are averages of two or three observations; other details as Table 2.

Bromide and Strychnine

Actions of bromide in the whole animal depend on chloride intake, and the object of the present experiments was to see whether absence of chloride made cerebral tissues more sensitive to bromide. Employing even 50 mM bromide, no greater sensitivity was found in the chloride-free medium (Table 4). This concentration had little if any effect on respiration in normal media, but higher concentrations were not examined, as nonspecific effects were feared. Absence of bromide sensitivity was found in normal respiration and in that stimulated by potassium salts or by applied pulses (for interaction between bromide ions and certain types of electrical pulses, see FORDA and MCILWAIN, 1953).

VOL 1 1956

Strychnine at 10^{-5}, 10^{-4}, or 10^{-3} M was without effect on the tissues' chloride; at 10^{-4} M respiration was slightly inhibited and glycolysis increased (Table 4), effects which became more marked at 10^{-3} M, but which in chloride-free solutions were the same as in chloride-containing ones. 10^{-4} M strychnine permitted nearly-normal respiratory responses to electrical pulses and to potassium salts, but markedly depressed the tissues' glycolytic response (Tables 3 and 4); actions which, again, were equal in chloride-free and in chloride-containing salines.

TABLE 4.—RESPIRATION WITH BROMIDE, STRYCHNINE, AND STIMULATING AGENTS

Applied agents		Period (min)	Respiration (μmoles O_2/g/h)	
Bromide or strychnine (S) (mM)	Others		Normal medium	Chloride-free
None	None	0–100	63	66
Br, 10, 20, 50	None	0–100	63–64	64–67
Br, 50	None	0–50	58 ± 3 (4)	65 ± 3 (4)
Br, 50	30 mM KNO_3	50–100	86 ± 4 (4)	95 ± 5 (4)
Br, 50	None	0–45	61 (3)	64 (3)
Br, 50	Electrical pulses	45–90	123 (3)	129 (3)
S, 0·01	None	0–100	64	66
S, 0·1	None	0–100	60	59
S, 1	None	0–100	48	43
S, 0·1	None	0–50	55	61
S, 0·1	30 mM KCl	50–100	85	104
S, 0·1	None	0–45	58	59
S, 0·1	Electrical pulses	45–90	85	94

Values are means, some followed by standard deviations and in parentheses the numbers of observations; others are averages of duplicates agreeing to within 5%.

Acetate Media

Experiments similar to certain of the preceding were carried out in media in which all chlorides were replaced not by nitrates but by acetates, the media being brought initially to pH 7.4 and buffered by the usual phosphates or bicarbonates. Whereas, as described above, findings in the chloride and nitrate media were similar to each other, those in the acetate medium were different (Table 5); these differences are ascribed to specific effects of the acetate ion rather than to lack of chloride. In the acetate media, respiration, though initially nearly normal, fell during two hours to less than half its normal value; glycolysis was high. Added potassium salts increased the already high glycolysis, but did not stimulate respiration. 50 mM bromide, curiously, opposed the fall in respiration.

DISCUSSION

In the living dog, with normally some 120 μequiv. of chloride/g, up to 44 μequiv./g can be replaced by nitrate without signs of toxicity (RAPOPORT and WEST, 1950; GREEN and HIATT, 1954). With separated tissues, more drastic changes can be made, and in the present experiments the normal 130 mM chloride of incubation fluids was reduced to below 1 mM without altering several metabolic characteristics of cerebral tissues, although incubation in such fluids reduced the tissues' chloride from 40 to 2 μmoles/g. This lower quantity of chloride was quite firmly held by the tissue; thus it persisted after washing and incubation in initially chloride-free media. Its mode of association with the tissue has not been studied; its quantity is too

TABLE 5.—RESPIRATION AND GLYCOLYSIS IN ACETATE MEDIA

Medium	Added agents (mM)	Period (min)	Respiration (μmoles O_2/g/h)	Glycolysis (μmoles/lactate/g/expt.)
Phosphate	None	0–60	51 (3)	148
		60–120	27	
Phosphate	None	0–60	45	153
	30 mM K^+	60–120	19	
Bicarbonate	None	0–120	—	124
Bicarbonate	None	0–60	—	167
	30 mM K^+	60–120	—	
Phosphate	10 mM Br^-	0–60	52	—
		60–120	24	—
Phosphate	20 mM Br^-	0–60	53	—
		60–120	27	—
Phosphate	50 mM Br^-	0–60	63	—
		60–120	60	—

Media contained acetate as the main anion, replacing the nitrate of the media detailed on p. 3; most values are the mean of three observations.

small to greatly affect calculations which have been made of intracellular/extracellular fluid volumes, based on chloride content (LOWRY *et al.*, 1954; MANERY, 1952; MCILWAIN, 1955).

Evidently the chloride retained by the tissue is adequate, if required, for the aspects of its metabolism which were studied, including any associated with metabolic response to electrical pulses. Thus the tissue is much less exacting in regard to chloride ions in its environment than it is in regard to sodium ions (GORE and MCILWAIN, 1952). Nor was the tissue sensitized to lack of chlorides by the presence of bromides or strychnine, in spite of the observations quoted in the beginning of the paper. Aspects of cerebral functioning affected by these substances appear not to be represented in the tissue as it has been studied in these experiments. In explanation

VOL 1 1956/

of this, it may be noted that phenomena concerned with synaptic transmission are not likely to be exhibited in this arrangement, which aims at directly applying added substances or exciting pulses to the greater part of the tissue.

SUMMARY

1. Chloride levels in different parts of the cerebral cortex of the guinea-pig were between 35 and 45 μmoles/g. Brief exposure of the separated tissue to chloride-free media lowered these values to 2 μmoles/g.

2. Tissue so treated, and placed in glucose-containing salines in which chlorides were replaced by nitrates, was capable of respiration and glycolysis at normal rates, and responded normally to ammonium and potassium salts and to electrical pulses.

3. Chloride-free media did not sensitize the tissues' metabolism to strychnine or to bromides.

4. When the chlorides of glucose salines were replaced by acetates, respiration was initially normal, but fell to half during two hours: it did not increase with increased concentrations of potassium salts. Glycolysis was high.

Acknowledgements—These studies have been generously supported from the Research Funds of the Bethlem Royal Hospital and the Maudsley Hospital, and we are grateful to the Board of Governors of the Hospital for the allocation for this purpose.

REFERENCES

Ayres P. J. W. and McIlwain H. (1953) *Biochem. J.* **55,** 607.
Barker S. B. and Summerson W. H. (1941) *J. Biol. Chem.* **138,** 535.
Conway E. J. (1947) *Microdiffusion Analysis and Volumetric Error*, London, Crosby Lockwood.
Coombs J. S., Eccles J. C., and Fatt P. (1953) *Austral. J. Sci.* **16,** 1.
Forda O. and McIlwain H. (1953) *Brit. J. Pharmacol.* **8,** 225.
Gore M. B. R. and McIlwain H. (1952) *J. Physiol.* **117,** 471.
Greene I. and Hiatt E. P. (1954) *Amer. J. Physiol.* **176,** 463.
LeBaron F. N. (1955) *Biochem. J.* **61,** 80.
Lewis J. L. and McIlwain H. (1954) *Biochem. J.* **57,** 680.
Lowry O. H., Roberts N. R., Leiner K. Y., Wu M. L., Farr A. L., and Alberts R. W. (1954) *J. Biol. Chem.* **207,** 39.
McIlwain H. (1951) *Biochem. J.* **49,** 382.
McIlwain H. (1953) *Biochem. J.* **53,** 403.
McIlwain H. (1955) *Biochemistry and the Central Nervous System*, London, Churchill.
McIlwain H. and Grinyer I. (1950) *Biochem. J.* **46,** 620.
McIlwain H. and Tresize A. M. (1956) *Biochem. J.* (in press).
Manery J. F. (1952) in *Biology of Mental Health and Disease*, New York, Hoeber, 124.
Rapoport S. and West C. D. (1950) *Amer. J. Physiol.* **162,** 668.
Rodnight R. and McIlwain H. (1954) *Biochem. J.* **57,** 649.
Schales O. and Schales S. S. (1941) *J. Biol. Chem.* **140,** 879.
Sunderman F. W. and Williams P. (1933) *J. Biol. Chem.* **102,** 279
Williams C. E., Siebert R. A., and Huggins R. A. (1954) *Amer. J. Physiol.* **177,** 87.

VOL. 1 56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 8-11, Pergamon Press Ltd., London.

DEPRESSION BY RESERPINE OF THE NORADRENALINE CONCENTRATION IN THE HYPOTHALAMUS OF THE CAT

MARGARETHE HOLZBAUER and MARTHE VOGT
Department of Pharmacology, University of Edinburgh

(*Received* 26 *November* 1955)

THE observation that reserpine causes a severe loss of 5-hydroxytryptamine from brain tissue (PLETSCHER, SHORE, and BRODIE, 1956; PAASONEN and VOGT, 1956) prompted the investigation of the effect of this drug on the noradrenaline content of brain. The region analysed was the hypothalamus, as its noradrenaline content is high and as the effect of reserpine on the 5-hydroxytryptamine of brain had been demonstrated on this region (PAASONEN and VOGT, 1956).

Cats were used in which the left adrenal gland had been denervated in an aseptic operation under ether between twelve and twenty days before the experiment. This procedure allowed simultaneous observation of the noradrenaline content of the hypothalamus and of any central stimulation of the sympathetic system produced by the drug; such stimulation would appear as a difference in the amount of medullary amines (adrenaline and noradrenaline) found in the innervated and the denervated gland. The methods used have all been reported (VOGT, 1954) with the exception of the use of pithed rats (SHIPLEY and TILDEN, 1947) for some of the assays of noradrenaline and adrenaline; this preparation gives a steadier baseline than the anaesthetized rat treated with hexamethonium. Control experiments showed that small doses of reserpine, such as might be present in the brain extracts, did not interfere with the assays.

The reserpine (Serpasil Ciba, ampoules of 2·5 mg/ml) was given i.p., and doses of the drug and duration of the experiments are shown in Table 1. They were the same as those found to release most of the 5-hydroxytryptamine from the hypothalamus of the dog. The results are presented in Table 1. Cats 1–3 belonged to a litter aged $4\frac{1}{2}$ months, cats 4–8 to a second litter of 14 months. Motility and posture were more seriously affected in the young than in the adult animals. The figures for the noradrenaline content of the hypothalamus are recorded in column 6; in column 7 they are expressed as percentages of a mean of 1·4 μg/g fresh tissue (range 0·90–2·08 μg) obtained previously for twenty normal cats of all ages. Column 8 expresses the findings in litter 2 as percentages of the noradrenaline concentration found in cat 8, an uninjected control of the same litter. These figures are probably more reliable than those of column 7, as the variation within litters has previously been found to be small (VOGT, 1954).

Irrespective of which calculation is chosen, the results leave no doubt that the injection of reserpine caused a loss in hypothalamic noradrenaline; the loss was very severe in the young kittens, less so in the adult animals. It was at least as great as that produced by large doses of morphine (VOGT, 1954). The effect of reserpine on the adrenal glands appears, however, to differ from that of morphine. After morphine,

VOL
1
1956/

TABLE 1. EFFECT OF ONE INTRAPERITONEAL INJECTION OF RESERPINE ON THE NORADRENALINE CONTENT OF THE HYPOTHALAMUS AND ON THE AMINE CONTENT OF DENERVATED AND INNERVATED ADRENAL MEDULLA

1	2		3	4	5	6	7	8	9	10	11	12	13
						Hypothalamic noradrenaline			Denervated adrenal		Innervated adrenal		
Exp. No.	Litter No.	Sex	Days since operation[1]	Dose of reserpine (mg/kg)	Interval between injection and dissection (hr)	μg/g fresh tissue	% of normal[2]	% of litter mate control	Noradrenaline (mg/g)	Adrenaline (mg/g)	Noradrenaline (mg/g)	Adrenaline (mg/g)	Total amines lost from innervated adrenal (denervated adrenal = 100)
1	1	♀	12	0·4	18	0·20	14·3	—	0·504	0·336	0·225	0·240	45
2	1	♀	12	0·4	18	0·22	15·7	—	0·225	0·360	0·108	0·168	53
3	1	♂	14	0·4	22	0·40	28·6	—	0·320	0·400	0·142	0·340	33
4	2	♂	14	0·4	18	0·80	57·2	38·4	0·556	0·522	0·040	0·090	88
5	2	♂	17	0·4	20	0·80	57·2	38·4	0·456	0·573	0·264	0·259	46
6	2	♂	17	0·4	20	0·86	61·5	41·3	0·270	0·192	0·192	0·168	22
7	2	♂	20	0·7	21	0·86	61·5	41·3	0·336	0·336	0·336	0·150	28
8	2	♂	20	0·0	—	2·08	—	—	0·420	0·456	0·380	0·390	12

[1] Unilateral adrenal denervation.

[2] Mean of 20 normal cats 1·4 μg/g fresh tissue.

the loss of medullary amines from the innervated adrenal was always very pronounced (greater than 60%), and appeared to be greatest when loss of hypothalamic noradrenaline was most severe. This correlation was not found after the use of reserpine. In litter 1, in which the loss of amines from the brain was very large, only about one-third to one-half of the medullary amines was lost from the innervated gland. In litter 2, all cats of which showed the same depletion of hypothalamic noradrenaline, the innervated adrenal of three animals had lost only 22–46% of the amount found in the denervated (resting) gland, whereas that of the fourth, an animal which was made very restless by the drug, had lost 88%. There was, however, no sign that the drug had released medullary amines by direct action on the adrenal, as would have been shown by a low amine content of the denervated glands. Their content varied from 0·46–1·08 mg/g, thus covering the same range as that previously found in denervated adrenals of cats not given reserpine (0·48–1·14 mg/g).

The interpretation of these results is not easy. If one assumes, as was done concerning the effect of reserpine on 5-hydroxytryptamine, that the phenomenon is one of release of the amine from the tissues perhaps comparable to the release of histamine by certain bases, this could take place without stimulation of the affected cells (in this case without stimulation of the sympathetic centres in the hypothalamus). In previous work on the effect of drugs on the noradrenaline content of the hypothalamus, depletion in the hypothalamus was only found (VOGT, 1954) when signs of sympathetic stimulation were present and evident both from clinical signs and from a fall in amine content of the innervated adrenal gland. The cats under reserpine exhibit no clinical signs of sympathetic stimulation, but that such stimulation takes place is shown by the difference in amine content of innervated and denervated adrenals, which, in cat 4, was as high as 88%, and in all animals exceeded the figures for untreated cats which range from zero to twenty per cent. Probably the sympathetic discharge is not noticed after reserpine because it is overshadowed by very powerful opposing effects, such as the relaxation of the nictitating membrane or the miosis, the origin of which is not at present known.

If there had been correlation between secretion of medullary amines and loss of hypothalamic noradrenaline, the whole effect on the hypothalamus could have been attributed to such sympathetic stimulation of, perhaps, a compensatory nature like that following an injection of insulin. The absence of such a correlation, however, may point to an essential difference in the mechanism underlying depletion of noradrenaline from the brain by reserpine and by drugs in which such a correlation exists. Whereas, when such a correlation is found, this suggests that the fall in the noradrenaline concentration is causally related to the central activity elicited by the drug, reserpine may produce this fall through a release mechanism dissociated from activity. Similarly, it was suggested that the falls in the 5-hydroxytryptamine concentration produced by reserpine and by amphetamine (PAASONEN and VOGT, 1956) are of a different nature, and that only the second may be an expression of the functional stimulation produced by the drug.

If a release mechanism is responsible for the fall in hypothalamic noradrenaline by reserpine, other tissues containing noradrenaline might be equally affected. Of these, only the denervated adrenal gland was tested, and no evidence was obtained that this contained less medullary amines than denervated glands of cats not given reserpine.

VOL
1
1956/

This last finding was confirmed by an experiment in which reserpine was given by close arterial injection into the coeliac artery of an anaesthetized vagotomized cat; the arterial blood pressure was recorded. In this preparation, liberation of hormone by direct stimulation of the adrenal medulla is detected by a rise in blood pressure which occurs immediately after the injection of a drug (FELDBERG and MINZ, 1932). Doses of reserpine rising from 1 to 500 μg were without effect on the blood pressure, whereas control injections of 6 to 10 mg KCl produced the usual pressor effects.

SUMMARY

The noradrenaline concentration of the cat's hypothalamus is reduced by one injection of reserpine (0·4 mg/kg) from its normal figure of 1·4 to between 0·20 and 0·86 μg/g fresh tissue. In all cats given reserpine the injection was followed by signs of sympathetic discharge as measured by a fall in medullary amines in the innervated adrenal in comparison to the denervated contralateral gland. There was, however, no correlation between the magnitude of hormonal discharge and the loss of hypothalamic noradrenaline.

VOL. 1 56/57

Acknowledgements—We are indebted to Messrs. Ciba Laboratories Ltd., Horsham, for a gift of Serpasil.

The work was carried out during the tenure by one of us (M. H.) of an I.C.I. Fellowship. M. V. is indebted to the Medical Research Council for a grant towards the expenses of this work.

REFERENCES

FELDBERG W. and MINZ B. (1932) *Arch. exp. Path. Pharmak.* **163,** 66.
PAASONEN M. K. and VOGT M. (1956) *J. Physiol.* in press.
PLETSCHER A., SHORE P. A., and BRODIE B. B. (1956) *J. Pharmacol.* **116,** in press.
SHIPLEY R. E. and TILDEN J. H. (1947) *Proc. Soc. exp. Biol. N.Y.* **64,** 453.
VOGT M. (1954) *J. Physiol.* **123,** 451.

Journal of Neurochemistry, 1956, Vol. 1, pp. 12–17, Pergamon Press Ltd., London.

ON THE PARTICIPATION OF THE GLUTAMIC ACID-GLUTAMINE SYSTEM IN METABOLIC PROCESSES IN THE RAT BRAIN DURING PHYSICAL EXERCISE

R. VRBA
Institute of Industrial Hygiene and Occupational Diseases, Prague

(*Received* 24 *October* 1955)

VOL
1
1956

IT is known that increased functional activity of the brain is associated with ammonia formation (BUDANOVA, 1950; PRAVDIČ-NEMINSKIJ, 1933; RICHTER and DAWSON, 1948; RÖSCH and TEKAMP, 1928; TASHIRO, 1922; VLADIMIROVA, 1953, 1954; VRBA, 1954b; WINTERSTEIN and HIRSCHBERG, 1925). Ammonia is toxic to nervous tissue (RICHTER and DAWSON, 1948; VLADIMIROVA, 1953) and produces a neuromuscular block even in small concentrations (GOODMANN and GILLMAN, 1941). For this reason the system which detoxicates the ammonia produced during activity is important for maintaining the working capacity of the tissue. This paper deals with the mechanism of detoxication of ammonia in the brain and the participation of glutamic acid in this process.

Swimming served as a method of producing increased activity of the brain in the experimental animals. In our previous experiments we found that during physical exercise the concentration of free ammonia in the brain of the experimental animals was not increased (VRBA, 1954b). The explanation of this apparent disagreement seems to be that ammonia produced in the brain during diffuse neuromuscular activity lasting for several hours is chemically bound to glutamic acid, and glutamine is produced. There is considerable evidence (ELLIOT, 1951; SPECK, 1949; WEIL-MALHERBE, 1950, 1952) of the activity in brain tissue of the enzymic system which forms glutamine. If this system is active *in vivo*, the ammonia produced is bound to glutamic acid, and, instead of an increased ammonia concentration, an increased concentration of free glutamine should occur. In previous experiments we found that this is actually the case, and we could demonstrate a significant increase of free glutamine in the brain during diffuse neuromuscular activity (VRBA, 1954b).

If the increased concentration of free glutamine in the brain is the result of an increased rate of amidation of glutamic acid, we should expect a simultaneous decrease in the concentration of free glutamic acid. This assumption was tested experimentally, and the results are given in the present paper. A preliminary account of these experiments has already been published (VRBA, 1955c).

METHODS

Arrangement of the experiment

The experiment was performed on seventy-two rats weighing 150–200 g each, which were divided into six experimental series. Each experimental series contained twelve rats of approximately the same weight and age, which had been kept for several days before the experiment in a common cage, to obtain more constant initial conditions for the experimental animals in each series. The twelve rats were divided into two groups containing six animals each. One group was made to swim

VOL.
1
56/57

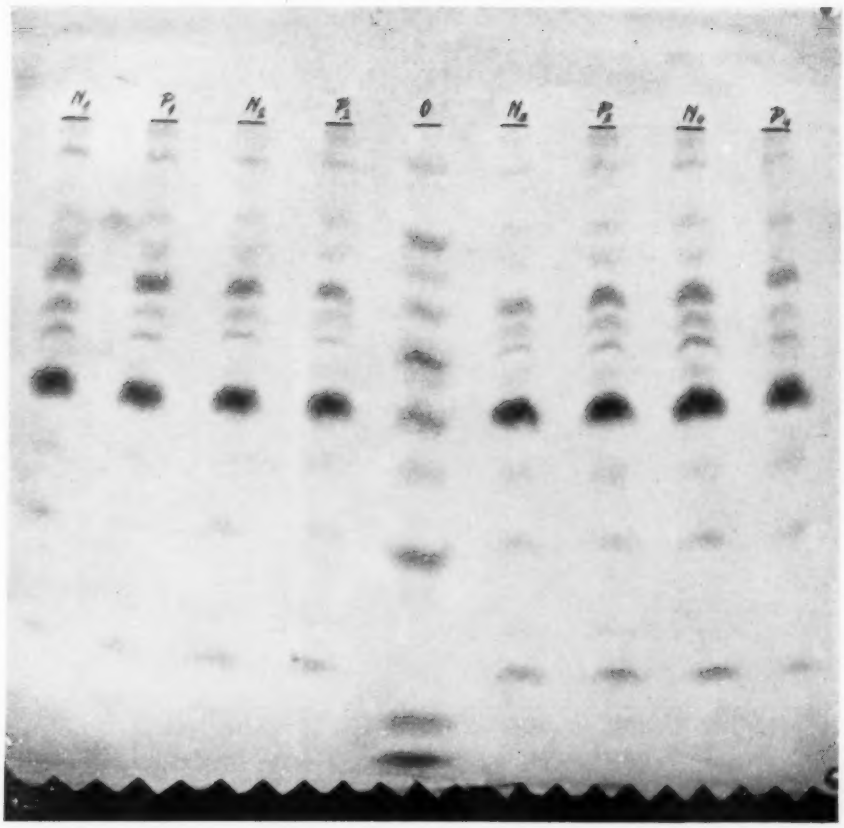


FIG. 1.—Experimental series No. 3. Samples of the control group are indicated by the letter *N*: samples of the fatigued group by the letter *P*. In the middle, a sample of ovalbumin hydrolysate is included (*O*), the composition of which is known and which serves to identify the amino acids.

facing p. 12

VOL
1
1956/

for 4½ h under the conditions described previously (VRBA, 1954a,b, 1955a). The second group served as controls. After swimming, the rats of the experimental and control group were dropped into liquid air. The frozen brains of the experimental animals were dissected out, ground to powder at a low temperature, and put in a weighed flask containing 100 ml of 12% trichloroacetic acid. The brains of the control animals were treated in the same way. The wet weight of the brains was determined by differential weighing. The trichloroacetic acid was then filtered off and the residual tissue was extracted three times more with 50 ml of 12% trichloroacetic acid solution. Combined trichloroacetic acid filtrates were then prepared for chromatographic analysis.

Preparation of samples for chromatography

The aqueous solution of free amino acids was prepared, with minor modifications, by the method of ANSELL and RICHTER (1954). Glutamine is labile in acid solution. For this reason the trichloroacetic acid extract of brain was prepared at a low temperature (0–3°C) and, immediately after, the TCA extract was immersed in a water bath at 70°C for seventy-five minutes. Under these conditions, the quantitative cyclization of glutamine to form pyrrolidone-carboxylic acid takes place, with the simultaneous splitting off of ammonia (ANSELL and RICHTER, 1954; HARRIS, 1943; RICHTER and DAWSON, 1948). Pyrrolidone-carboxylic acid is a relatively stable compound.

After this procedure had been completed, the trichloroacetic acid was removed by shaking the TCA extract with 250 ml ether in a separating funnel, and this operation was repeated four times (ANSELL and RICHTER, 1954). The last traces of trichloroacetic acid remaining in the aqueous solution were removed by immersing the solution in a boiling-water bath for two hours. The aqueous solution of free amino acids was evaporated *in vacuo* to dryness. The residue thus obtained, which was not completely soluble in water, was dissolved by extracting four times with 10 ml of water, and the insoluble residue was centrifuged off. The combined supernatants were again evaporated *in vacuo* to dryness. The residue was dissolved in a measured amount of water corresponding to 0·5 ml per g fresh brain-tissue. The amino acid solution thus obtained was then applied directly to the chromatographic paper.

Chromatography and quantitative evaluation of chromatograms

The work of FISCHER and DORFEL (1953) showed that unidimensional chromatography is sufficient for the quantitative determination of amino acids. We therefore used unidimensional chromatography, with minor modifications according to KEIL (USAKOV, 1950). The amino acids from the control and fatigued groups of each series were applied to Whatman No. 1 paper (48 × 48 cm), using four samples from the control group alternatively with four from the fatigued group of the same series. The samples were applied on the starting line (10 cm from the edge) using 15 µl, which corresponded to 30 mg of fresh brain-tissue, in the form of a thin line 15 mm long. No de-salting procedure was necessary. For separation, a mixture of butanol–acetic acid–water was used. Chromatograms were developed with 1% ninhydrin solution in dry acetone. The chromatograms were immersed in this solution, and after they had been allowed to dry in the air they were dried further for 30 min at 60°C, and were photographed on a microfilm of 1 × 1 cm. The films were evaluated microphotometrically on the Soviet microphotometer MF-2. Chromatographic separation, photography, and evaluation of the chromatograms were performed with small modifications by KEIL's method (KEIL, 1954).

The figures obtained by the microphotometric evaluation of the chromatograms were plotted on co-ordinate paper (841 × 1189 mm). In this way, an amino acid "spectrum" was obtained. Each maximum on the curve corresponds to one amino acid, the area below this maximum being proportional to the concentration of this amino acid. The areas of the curves of glutamic acid were measured planimetrically. Curves were drawn on the large scale mentioned to render the planimetric evaluation more accurate.

Each sample of the control group (pooled sample of six brains) was chromatographed four times on one paper, and on the same paper the pooled sample of six brains of the respective fatigued group was chromatographed four times. In this way, four curves of the control group and four curves of the respective fatigued group were obtained. The area below the maximum corresponding to glutamic acid was therefore determined four times, and the mean of these four values was compared with the mean value of the corresponding fatigued group, obtained in the same way.

VOL. 1 56/57

Statistical evaluation of results

Each experimental series contained two groups, the fatigued group and the control group. The glutamic acid concentration was proportional to the area under the curves. The difference in the glutamic acid area of the control group and that of the corresponding fatigued group expressed in square centimetres served as a basis for the statistical calculation. The use of STUDENT's t test of the difference of means between paired observations was justified under these conditions.

$$t = \frac{\text{mean difference of glutamic acid in square centimetres between the control and the fatigued group}}{\text{mean error of mean difference}}$$

RESULTS AND DISCUSSION

Fig. 2 shows a typical microphotometric evaluation (columns N_3 and P_3 on chromatogram No. 5). In the brain of the fatigued group the glutamic acid content was always smaller than that of the control group. This was observed in all six experimental series. The results of the microphotometric evaluations shown in

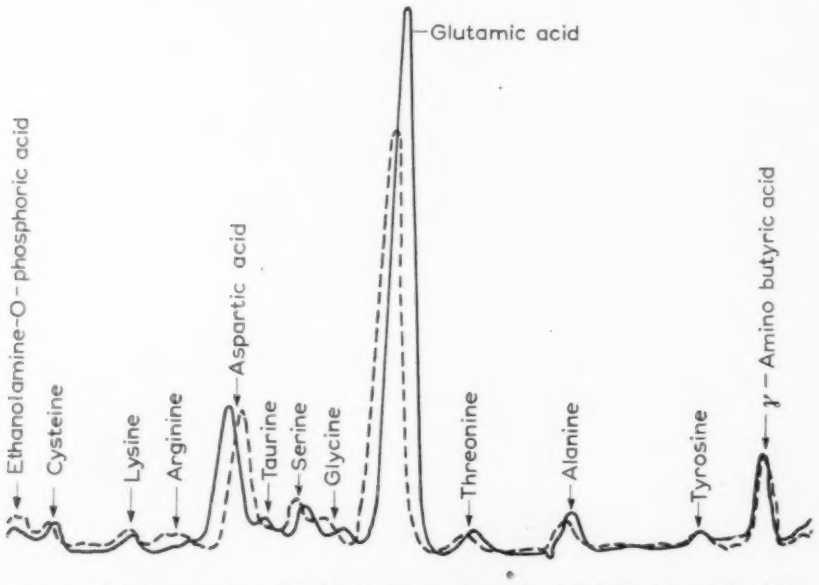


FIG. 2.—Microphotometric evaluation of chromatogram No. 5 columns N_3 and P_3. The "spectrum" of free amino acids from the brains of the control group is marked with an unbroken line. The dotted line corresponds to free amino acids in the brain after 4½-h swimming. The area below each maximum is related to the concentration of the corresponding amino acid. In the free (acid-soluble) fraction of the brains of the fatigued group of rats, the concentration of glutamic acid was always lower than in the corresponding control group.

Table 1 give evidence that diffuse neuromuscular activity (4½ h swimming) is associated with a decrease in the concentration of free glutamic acid in the rat brain. As for the other amino acids, their concentration either did not change at all during physical exercise, or only minor variations were observed, as is usual with biological material. A definite and regular decrease could only be demonstrated in the case of glutamic acid.

On the basis of experimental results published previously (VRBA, 1955a,b), we

TABLE 1.—EFFECT OF PHYSICAL EXERTION ON THE CONCENTRATION OF GLUTAMIC ACID IN THE RAT BRAIN

No. of series	Control group (A) cm^2	Fatigued group (B) cm^2	Difference
1	161·9	112·4	−49·5
2	137·1	99·0	−38·1
3	132·9	119·3	−13·6
4	221·0	138·2	−82·8
5	197·1	146·2	−50·9
6	243·8	181·3	−62·5
			Mean −49·6 ± 9·5

$t = 5{\cdot}2;\ P < 0{\cdot}01$

(Each value in the table expresses the area of the spot of glutamic acid in cm^2 from the pooled sample of six brains of the control or the fatigued group, and is the average of four parallel determinations.)

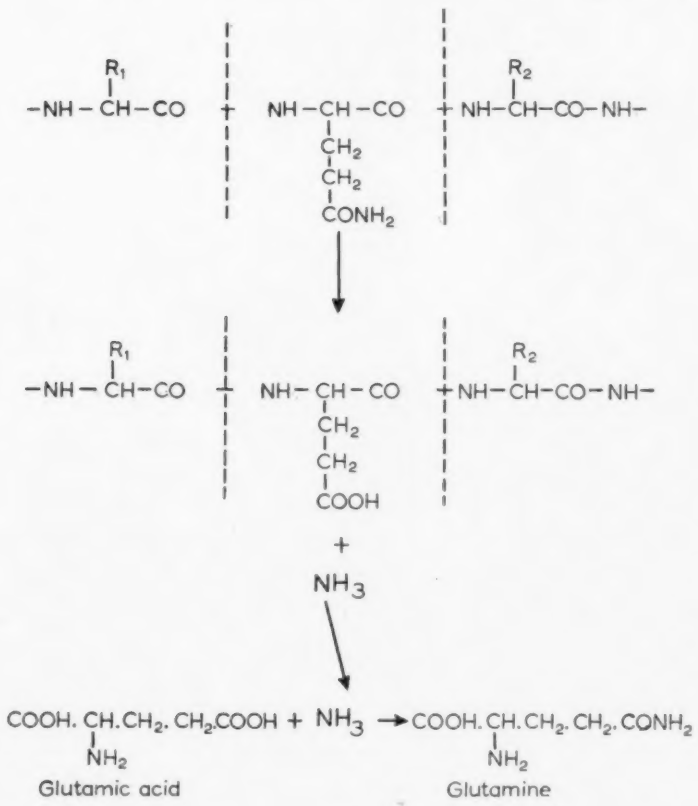


FIG. 3.

concluded that during the activity of the brain associated with diffuse neuromuscular activity, the following metabolic processes occur in the brain (Fig. 3):

This scheme is based on the following facts:

1. During increased activity of the rat brain a decrease of the amide group in the brain protein fraction takes place (VRBA, 1955a,b). This amide nitrogen comes for the most part from the protein-bound glutamine (RICHTER, 1955; VRBA, 1955a).

2. During functional activity of nervous tissue, ammonia is formed (BENITEZ *et al.*, 1954; BUDANOVA, 1950; PRAVDIČ-NEMINSKIJ, 1933; RICHTER and DAWSON, 1948; RÖSCH and TEKAMP, 1928; TASHIRO, 1922; VLADIMIROVA, 1953, 1954; VRBA, 1954b; WINTERSTEIN and HIRSCHBERG, 1925).

3. During the activity of the brain in association with physical exercise, there is an increase in the concentration of free glutamine in the brain (VRBA, 1954b).

4. This scheme fits in with the fact, described by other authors, that functional activity of nervous tissue is associated with changes in the structure of the tissue proteins (FRANK *et al.*, 1954; NASONOV and ALEKSANDROV, 1941; ROMANOV, 1953; ROMANOV, 1955; SEGAL, 1953/1954; UŠAKOV, 1950, 1954; VRBA, 1955b; ZARAKOVSKIJ and LEVIN, 1953).

One of the further assumptions of this scheme is that, parallel with the increase in the concentration of free glutamine in the brain, the concentration of free glutamic acid during physical exercise should decrease. The correctness of this assumption was demonstrated experimentally in the present work.

It should be emphasized that the participation of glutamic acid in the processes associated with functional activity of the brain are not restricted to the above-mentioned process.

SUMMARY

Changes in the concentration of free glutamic acid in the rat brain during diffuse neuromuscular activity were observed. It was found that during physical exertion by swimming, a statistically significant decrease in the concentration of free glutamic acid took place ($P < 0{\cdot}01$). This experimental finding is in agreement with the previously described scheme, according to which a splitting-off of the amide group in the brain proteins occurs during functional activity of the brain. The ammonia formed during this process combines with glutamic acid, the process resulting in an increase in the concentration of free glutamine in the brain. The concentration of free glutamine in the brain increased at the expense of free glutamic acid. Glutamic acid was the only free amino acid in the brain in which a regular decrease in concentration during physical exertion could be shown by the method of chromatographic analysis. It is concluded that the decrease in the concentration of glutamic acid under these conditions is related to the specific role of glutamic acid in the brain.

REFERENCES

ANSELL G. B. and RICHTER D. (1954) *Biochem. J.* **57,** 70.

BENITEZ D., PSCHEIDT G. R., and STONE W. E. (1954) *Amer. J. Physiol.* **176,** 488.

BUDANOVA A. N. (1950) *Dokl. Akad. Nauk S.S.S.R.* **75,** 875.

ELLIOT W. H. (1951) *Biochem. J.* **49,** 106.

FISCHER F. G. and DÖRFEL H. (1953) *Biochem. Z.* **324,** 544.

FRANK G. M., ALADŽALOVA N. A., KAJUŠIN B. P., and LJUDKOVSKAJA R. G. (1954) in *Biochimija Nervnoj Sistemy. Izd. Akad. Nauk Ukr.S.S.R., Kijev.*

GOODMAN L. and GILLMAN A. (1941) *The Pharmacological Basis of Therapeutics*, New York.
HARRIS M. M. (1943) *J. Clin. Ivest.* **22**, 569.
KEIL B. (1954) *Collection Czechoslov. Chem. Com.* **19**, 1006.
NASONOV D. N. and ALEKSANDROV V. JA. (1941) *Reakcija Živogo Věščestva na Vnešnije Vozdejstija*. Izd. Akad. Nauk S.S.S.R.
PRAVDIČ-NEMINSKIJ V. V. (1933) *Arch. biol. nauk S.S.S.R.* **33**, 121.
RICHTER D. and DAWSON R. M. C. (1948) *J. Biol. Chem.* **176**, 1199.
RICHTER D. (1955) Brain metabolism, *I. International Neurochemical Symposium*, Academic Press New York.
ROMANOV S. N. (1953) *Dokl. Akad. Nauk S.S.S.R.* **90**, 117.
ROMANOV S. N. (1955) *VIII. Vsesojuznyj sjezd Fysiologov, Farmakologov i Biochimikov. Tezisy Dokladov*. Izd. Akad. Nauk S.S.S.R., Moskva.
RÖSCH H. and TEKAMP W. (1928) *Z. physiol. Chem.* **175**, 158.
SEGAL J. (1953/1954) *Wiss. Z. Humboldt-Univ. Berlin* **3**, 243.
SPECK J. F. (1949) *J. Biol. Chem.* **179**, 1405.
TASHIRO S. (1922) *Amer. J. Physiol.* **60**, 519.
UŠAKOV V. P. (1950) *Dokl. Akad. Nauk S.S.S.R.* **71**, 205.
UŠAKOV V. P. (1954) *Uspechi sovr. biol. U.S.S.R.* **38**, 294.
VLADIMIROVA E. A. (1953) in *Voprosy Fiziologii i Morfologii Centralnoj Nervnoj Sistemy*. Pod redakciej K. M. BYKOVA. Izd. Akad. Nauk U.S.S.R., Moskva (1953).
VLADIMIROVA E. A. (1954) in *Biochimija Nervnoj Sistemy*. Izd. Akad. Nauk Ukr.S.S.R., Kijev.
VRBA R. (1954a) *Physiol. Bohemoslov.* **3**, 122.
VRBA R. (1954b) *Physiol. Bohemoslov.* **3**, 394.
VRBA R. (1955a) *Physiol. Bohemoslov.* **4**, 397.
VRBA R. (1955b) *Nature* **116**, 117.
VRBA R. (1955c) *Nature* **116**, 1258.
WEIL-MALHERBE H. (1950) *Physiol. Rev.* **30**, 548.
WEIL-MALHERBE H. (1952) *Biochem. Soc. Symp.* No. 8, 16.
WINTERSTEIN H. and HIRSCHBERG E. (1925) *Biochem. Z.* **156**, 138.
ZARAKOVSKIJ G. M. and LEVIN S. V. (1953) *Fiziol. ž. S.S.S.R.* **39**, 81.

VOL.
1
56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 18–21, Pergamon Press Ltd., London.

PURIFICATION AND PROPERTIES OF SPHINGOSINE

ROSCOE O. BRADY and ROBERT MAIN BURTON
Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness,
National Institutes of Health, United States Public Health Service,
Department of Health, Education, and Welfare, Bethesda, Maryland

(*Received* 27 *December* 1955)

IN the course of studies on the biochemistry of the complex lipids of nerve tissue, it has become necessary to have available analytically pure sphingosine for use as substrate in enzyme systems and for the organic synthesis of labelled lipids. We wish to describe a new method for the isolation of pure sphingosine base, a procedure for the analysis of minute quantities of this material, and some of its chemical and physical properties.

VOL
1
1956/

PREPARATION OF SPHINGOSINE

The procedure described by CARTER (1947) was followed for the initial hydrolysis of cerebrosides. All distillations were performed under nitrogen. The starting material was a mixture of cerebrosides obtained as a gift from Armour and Company (Lot AE-500926). The sphingosine sulphate obtained on hydrolysis was taken up in 100 volumes of methanol at room temperature and decoloured with a small amount of Norit-A. The methanol was removed under nitrogen, and colourless sphingosine sulphate (m.p. 149–152° cor.) was obtained. Elementary analysis revealed: calculated, C 62·03, H 10·99, N 4·02, S 4·60; found, C 61·71, H 11·03, N 4·35, S 4·47. Fig. 1 shows the X-ray diffraction spectrum of this compound.* 500 mg of the sulphate were suspended in 50 ml of water saturated with solid NaCl. The mixture was made strongly alkaline with NaOH. The free base was extracted with 250 ml of 2 per cent isoamyl alcohol in heptane† by shaking for 1 h. The mixture was clarified by centrifugation, and the isoamyl alcohol-heptane layer removed and evaporated to dryness at reduced pressure under nitrogen. The free sphingosine was recrystallized twice from petroleum ether. The solvent was removed and the sphingosine was dried *in vacuo* over phosphorus pentoxide.

PROPERTIES OF SPHINGOSINE

The purified sphingosine thus obtained was a stable white waxy solid which melted at 82·5–83°. This figure agrees with the value reported by KLENK and DIEBOLD (1931). The sphingosine exhibited optical activity, $[\alpha]_D^{25} = +3°$ cm² per g (26 mg in 2 ml of pyridine). The product was readily converted into triacetyl sphingosine, which melted between 99·5 and 100°. Elementary analysis of the sphingosine was determined. Calculated, C 72·19, H 12·45, N 4·68; found, C 72·02, H 12·46, N 4·52.

* The authors are indebted to Dr. GUNTHER L. EICHHORN of the National Institute of Mental Health for assistance in obtaining the X-ray diffraction spectrum, and to Dr. WARREN L. PETICOLAS and Mr. H. FRANKLIN BYERS of the National Heart Institute for the optical rotation and infra-red spectrum.

† The solvents were previously washed with one-third volume of N NaOH followed by a similar quantity of N HCl, and then with water.

VOL.
1
56/57

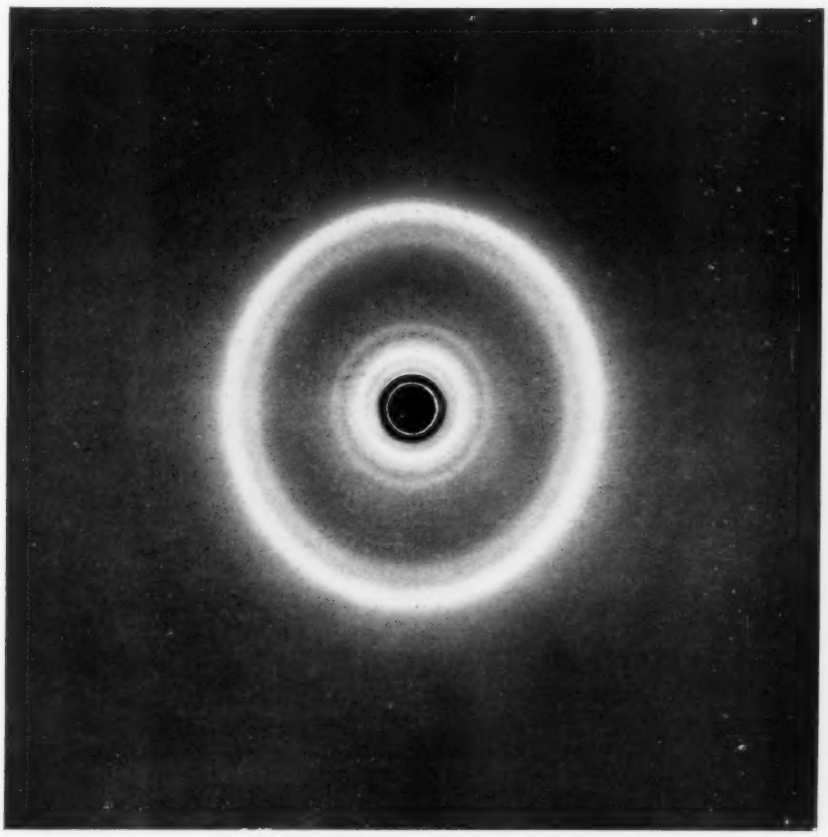


FIG. 1.—X-ray diffraction spectrum of sphingosine sulphate. Exposure, 5 h, copper K_α radiation in a Unicam camera of 3-cm radius. The strongest lines in order of decreasing intensity occur at 4·12, 14·7, 4·70, 9·44, 18·5, and 12·7 Å.

facing p. 18

VOL
1
1956/

Bromination of sphingosine was performed by a semimicro adaptation of the standard procedure for determining the uptake of bromine by Mr. J. MARTINEZ of this laboratory. The results of the bromination of a sample of sphingosine are shown in Table 1.

A stable solution of sphingosine hydrochloride can be obtained by treating a suspension of sphingosine in water with exactly one equivalent of HCl followed by gentle warming. Employing this technique, stable solutions of sphingosine hydrochloride as high as 0·03 M have been prepared. The ultra-violet absorption spectrum

TABLE 1.—BROMINATION OF SPHINGOSINE

Sample		Bromine			
mg	μmoles	Total absorbed (μmoles)	By substitution (μmoles)	By addition (μmoles)	Theoretical (per cent)
16·96	56·75	57·65	0·0	57·65	101·6
14·92	49·90	50·35	0·0	50·35	100·8

The reaction was carried out in 60-ml reagent bottles fitted with ground-glass droppers through which the bromine and potassium iodide reagents were added, maintaining a liquid seal during these additions. The mixtures were titrated with 0·0935 N $Na_2S_2O_3$ solution, a microburette with a total capacity of 1·0 ml being used.

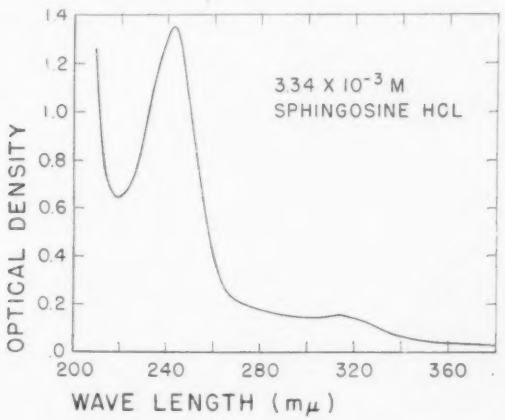


FIG. 2.—Ultra-violet absorption spectrum of sphingosine hydrochloride.

of sphingosine hydrochloride in water is shown in Fig. 2. A sharp absorption maximum is seen at 242 mu, and at this wavelength the extinction coefficient is $4·0 \times 10^5$ cm^2 per mole. The infra-red spectrum of sphingosine is shown in Fig. 3. Characteristic peaks are seen in the region of the hydroxyl (2·96 μ), carbon-hydrogen (3·42 μ), olefin (6·0 μ), amino (6·3 μ), and methylene (6·84 μ) stretching frequencies. The spectrum corresponds favourably with that reported by MISLOW (1952) for an uncharacterized sample of sphingosine prepared by I. ZABIN.

MICROANALYTICAL DETERMINATION OF SPHINGOSINE

Since sphingosine contains two hydroxyl groups vicinal to a secondary amino group and accordingly is oxidizable by periodate, a method for the quantitative estimation of sphingosine was developed, utilizing the change in optical density of a solution of periodate which decreases as the reaction proceeds (MACGEE and DOUDOROFF, 1954). From 0·05 to 0·5 μmole of sphingosine was dissolved in 2·0 ml

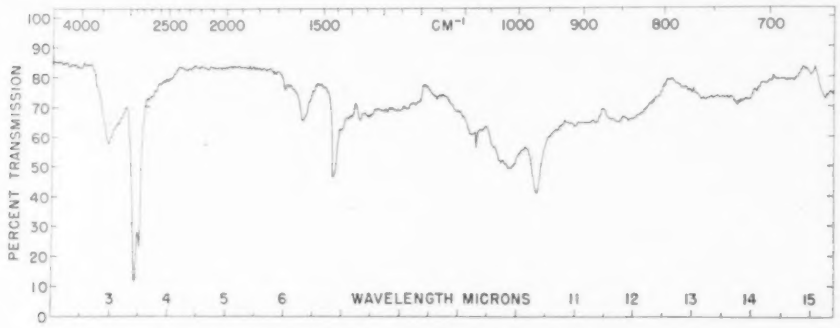


FIG. 3.—Infra-red spectrum of sphingosine in chloroform.

of 2 per cent isoamyl alcohol in heptane and added to a 12 ml glass-stoppered centrifuge tube containing 1·5 μmoles of sodium periodate in 1·65 ml of 0·1 M potassium acetate buffer (pH 3·8). The stoppers were greased with a small amount of silicone grease and inverted and shaken for 40 min in an International size-2 bottle shaker. After the shaking was completed, the isoamyl alcohol-heptane layer was removed by aspiration and the tubes centrifuged at 4000 r.p.m. for 5 min to clarify any residual emulsion. The final traces of isoamyl alcohol-heptane were removed by aspiration, and the periodate consumption was measured spectrophotometrically at 260 mu. Mannitol was employed as the primary standard. Reproducible values were obtained

TABLE 2.—DETERMINATION OF SPHINGOSINE BY PERIODATE OXIDATION

Exp. No.	Sphingosine added (μmoles)	Periodate consumed		Formaldehyde produced	
		Found (μmoles)	Theory (per cent)	Found (μmoles)	Theory (per cent)
1	0·260	0·431	83	0·242	93
	0·260	0·464	89	0·247	95
	0·260	0·408	79	0·240	92
2	0·270	0·480	89	0·267	99
	0·270	0·491	91	0·273	101
	0·270	0·507	94	0·279	103
3	0·135	0·251	93	0·139	103
	0·135	0·230	85	0·130	96
	0·135	0·259	96	0·132	98

See text for description of method.

for samples of sphingosine between 0·05 and 0·5 μmole. To insure maximal accuracy, the quantity of formaldehyde produced by periodate oxidation was determined. The amount of formaldehyde in a 0·5-ml aliquot of the aqueous solution was measured by treating the mixture with 5 ml of chromotropic acid reagent (BURTON, 1956), after the excess periodate had been destroyed with arsenite. The reliability of this method is illustrated in Table 2.

DISCUSSION

WITTENBERG (1955) has recently described a method for the separation of sphingosine and related compounds by reversed-phase partition chromatography of the respective N-succinyl derivatives. The method was reported to be reliable in quantities ranging from 3 to 100 μmoles. For our studies of the behaviour of sphingosine in various enzyme systems, we required a method capable of yielding reproducible values at much lower concentrations. Such a method was obtained by partitioning between a salt-saturated aqueous phase and a 2 per cent isoamyl alcohol-heptane mixture followed by periodate oxidation, and determining the formaldehyde liberated in the reaction. The method has been used with success for metabolic studies employing slice- and minced-tissue preparations as well as with a partially purified enzyme system. The method has not proved to be satisfactory for the recovery of sphingosine from crude homogenates of tissues.

Probably the outstanding advantage of this method is the fact that analytically pure, stable sphingosine can easily be prepared in good yield. This material has been characterized and used successfully for the starting material for the synthesis of C^{14}-labelled ceramides, whose chemical and biological properties will be reported in subsequent communications.

SUMMARY

A method for the preparation of sphingosine is described and some of the characteristics and properties of the purified compound are reported. A microanalytical estimation of sphingosine based on periodate oxidation followed by determination of the formaldehyde produced has been found to give satisfactory reproducible results.

REFERENCES

BURTON R. M. (1956) *Methods in Enzymology 3* (Colowick and Kaplan, New York). In press.
CARTER H. E., NORRIS W. P., GLICK F. J., PHILLIPS G. E., and HARRIS R. (1947) *J. Biol. Chem.* **170,** 269.
KLENK E. and DIEBOLD W. (1931) *Z. Physiol. Chem.* **198,** 25.
MACGEE J. and DOUDOROFF M. (1955) *J. Biol. Chem.* **210,** 617.
MISLOW K. (1952) *J. Amer. Chem. Soc.* **74,** 5155.
WITTENBERG J. B. (1955) *J. Biol. Chem.* **216,** 379.

VOL. 1 56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 22-31, Pergamon Press Ltd., London.

THE OCCURRENCE AND DISTRIBUTION OF 5-HYDROXYTRYPTAMINE (ENTERAMINE) IN THE CENTRAL NERVOUS SYSTEM OF VERTEBRATES

P. CORREALE
Institute of Pharmacology, University of Bari, Bari, Italy

(*Received* 10 *February* 1956)

VOL
1
1956

IT is now well established that the central nervous system of mammals as well as certain ganglia and peripheral nerves of invertebrates contain detectable quantities of 5-hydroxytryptamine (5-HT).

AMIN, CRAWFORD, and GADDUM (1954) first found that acetone extracts from various parts of the central nervous system of the dog stimulated the atropinized estrous uterus of the rat. The contraction produced (spasmogenic effect) corresponded, per gram of fresh tissue, to that provoked by 0·01–0·35 μg of 5-HT. The area richest in 5-HT was the hypothalamus, followed by the area postrema, the mid-brain, and other areas of grey matter. 5-HT was not found in the cerebellum, the white matter, or peripheral nerves.

Similar results were obtained by TWAROG and PAGE (1953), ZETLER and SCHLOSSER (1954) and, quite recently, by GARVEN (1955). The first investigators found that the 5-HT content of the whole brain of the dog is 0·1–0·36 μg/g and that of the rat 0·24 μg/g. The isolated heart of *Venus mercenaria* (which is five to ten-fold more sensitive to bufotenine and twice as sensitive to N-methyl-5-HT as to 5-HT itself) was used as a test-object. ZETLER and SCHLOSSER (1954), also using a molluscan heart preparation, the isolated heart of *Helix pomatia*, estimated the 5-HT content of ala cinerea and trigonum hypoglossi of the ox brain as 0·07 μg/g. Finally, GARVEN (1955), working on the rat uterus preparation, gives the following 5-HT values for the rabbit brain: hypothalamus 0·40 μg/g, mid-brain 0·37 μg/g, olfactory bulbs 0·17 μg/g, cerebellum 0·03 μg/g.

In the invertebrates, FLOREY and FLOREY (1953, 1954) found a substance biologically indistinguishable from 5-HT in aqueous extracts of cuttle-fish and crab ganglia (20–82 μg per gram of dry weight) as well as in extracts of the nerves of crab legs (30–66 μg/g). WELSH (1953, 1955) has confirmed these data, working with extracts of molluscan ganglia and heart. In ganglia of *Venus mercenaria* (pooled cerebro-pleural, visceral, and pedal ganglia) the ratio of 5-HT (about 15 μg/g fresh tissue) to acetylcholine was found to be 4 : 1, in ganglia of *Busycon canaliculatum* about 1 : 1. The occurrence of 5-HT in ganglia and nerves of molluscs has been again established by ERSPAMER (1955), both by bioassay and by paper chromatography (*Octopus vulgaris*: 0·3–2·2 μg 5-HT per gram fresh tissue). This investigator found, in contrast, unexpectedly low or even doubtful values in the nervous tissue of crustaceans (*Palinurus vulgaris*: $<$0·05 μg/g, both in ganglia and in leg nerves) and failed to detect 5-HT in the cerebral ganglia of an insect, *Locusta migratoria*, as well as in the ganglionic ventral chain of *Hirudo medicinalis* ($<$0·05 μg/g), in spite

of the presence in this tissue of chromaffin cells which are, in many respects, similar to the enterochromaffin cells.

Because we attribute great importance to comparative anatomy and biochemistry, as a key to the better understanding of the physiological significance of 5-HT, we have begun an extensive study of the occurrence and distribution of 5-HT in the nervous tissue throughout the vertebrate and invertebrate series. This paper gives an account of the first results of this study.

METHODS

Preparation of brain extracts. With the exception of dogs and cats, which were anaesthetized with pentobarbital (20 mg/kg i.v.), all other animals were used unanaesthetized. The brains were removed immediately after the animals had been killed by bleeding or, in the slaughter-house, by a pistol shot. The various parts were dissected out, weighed, cut up, and placed in acetone (4-5 ml per gram of tissue). After standing overnight, the acetone was decanted and the tissue re-extracted with another 3–4 ml of 80% acetone per gram of tissue. The combined filtrates were kept in a refrigerator until used. They were then evaporated *in vacuo*, and the residue taken up in a small amount of saline. This extraction procedure has been used in our laboratory since 1940.

In the case of extracts destined for paper chromatography, the residue was taken up in 2 ml of water and then shaken two or three times with light petroleum to remove lipids. The aqueous phase was evaporated to dryness *in vacuo*, and the residue taken up in 1 ml of 50% alcohol + 10 ml acetone. After standing overnight in a refrigerator, the liquid was filtered, the small precipitate washed with 95% acetone, and the combined filtrates reduced, *in vacuo*, to the desired volume (1 ml liquid = 10–50 g tissue).

Bioassay. The estrous uterus of the rat, suspended in an aerated 10 ml bath of Tyrode solution containing atropine (10^{-6}), at 30–31° C, was used throughout the experiments. A limited number of assays was carried out on the guinea-pig's ileum suspended in a bath containing atropine and mepyramine, in order to detect substance P. Uterine horns removed from ovariectomized rats gave more uniform and satisfactory results than those removed from non-ovariectomized animals pre-treated with stilbestrol. Although for the quantitative estimation of 5-HT all aspects of the uterus response were considered, particular attention was paid to the behaviour of the tone, as recorded for 3–4 min. The height of the first contraction following introduction into the nutrient bath of pure 5-HT or of the extract to be tested is, in our opinion, of secondary importance. We wish to lay particular emphasis on this point, since it is doubtful whether our results are in every case comparable with those obtained by other criteria.

The standard was the 5-HT creatinine sulphate synthetized in the Farmitalia Research Laboratories; however, the values were always expressed as 5-HT base.

Paper chromatography. The ascending unidimensional technique on Whatman No. 1 paper was always employed. Solvents: *n*-butanol + acetic acid + water (4 : 1 : 5); *n*-butanol + methylamine 25–30% (8 : 3). Developing reagents: HEINRICH and SCHULER'S NNCD reagent (4-nitro-2-chloro-1-diazobenzene-α-naphthalene sulphuric acid) freshly dissolved, 1–3 per thousand, in 0·1 N HCl; freshly prepared solution of diazotized *p*-nitroaniline and 10% sodium carbonate.

When paper chromatography was used to separate 5-HT from interfering substances, at least three applications were made on every paper sheet. One consisted of ten 0·01 ml drops of the brain extract under investigation (a), the other two—(b), (c)—of the same quantity of extract plus 1–3 μg 5-HT. After the chromatograms were run for 18–20 h, the paper sheets were dried and then divided in three in the direction of the solvent flow. Strip (c) was sprayed with the diazonium salt solution to determine exactly the position of the 5-HT spot; the corresponding areas of strips (a) and (b) were separately cut out and eluted with saline. Eluates of strip (a) served to determine the 5-HT activity of the brain extract alone, eluate of strip (b) to determine the recovery of added 5-HT.

Substances interfering in the bioassay of 5-HT. This point has been thoroughly investigated by ERSPAMER (1954) and by AMIN, CRAWFORD, and GADDUM (1954), and we refer to their papers for further details.

Brain extracts prepared with 80% acetone contain, in addition to 5-HT, other uterus-stimulating substances, such as choline esters, adenosine compounds, substance P, and posterior pituitary

hormone, as well as uterus-inhibiting substances such as adrenaline, noradrenaline, and histamine. The possibility cannot be ruled out, especially in lower vertebrates, of the presence of additional unknown active products.

Histamine is of practically no importance, and the interference of choline esters may be easily neutralized by atropine. Interference by the other active products can hardly be avoided if they are present in sufficient amounts and the 5-HT content is comparatively low, as is usual in brain extract.

VOGT (1954) has shown that several parts of the central nervous system contain sympathin (10% adrenaline + 90% noradrenaline) in quantities greater than 0·1 μg/g. The substance is completely extracted by 80% acetone. According to AMIN, CRAWFORD, and GADDUM (1954), the assay results for 5-HT are unreliable when adrenaline and noradrenaline are present in the extracts in amounts ten times smaller and ten times greater, respectively, than 5-HT.

Adenosine compounds may be present in tissues in amounts equivalent to 100–1000 μg or more of adenosine per gram of tissue. In experiments carried out by ERSPAMER (1954), 1 μg of 5-HT was roughly equivalent, on the rat-uterus preparation, to 2–5 mg of adenosinetriphosphoric acid. We have not investigated how much of the adenosine compounds passes into 80% acetone, but it seems unlikely that these products contribute to the contraction produced by brain extracts on the rat uterus.

AMIN, CRAWFORD, and GADDUM (1954) found that 80% acetone extracted 29% of substance P from a horse's intestine. If this is true even for the brain, it is clear that substance P may in some cases interfere seriously with the estimate of 5-HT. Data on the concentration of substance P in nervous tissue are given by PERNOW (1955) and by others. According to ERSPAMER (1954) and to AMIN, CRAWFORD, and GADDUM (1954), 1 μg of 5-HT is roughly equivalent to 60–100 units of substance P.

The posterior pituitary hormone very powerfully stimulates the estrous uterus of the rat: 1 unit is equivalent to 100 μg of 5-HT. In practice, the possibility that estimations of 5-HT may be complicated by the presence of oxytocin exists only for the hypothalamus and, of course, only for the quantities of hormone which are extracted by 80% acetone.

In order to minimize the hardly avoidable interferences we have just discussed, three procedures were tried:

(a) Extraction of the nervous tissue with 20 vol of acetone, instead of our usual 4 vol, as suggested by AMIN, CRAWFORD, and GADDUM (1954). The advantage of this method would be that it eliminates the interference of substances insoluble in 95% acetone, such as adenine compounds, substance P, posterior pituitary hormone, and possibly other polypeptides active on the smooth muscle. Unfortunately, 95% acetone did not appear, in our control experiments, to extract all the 5-HT contained in the tissues, and the method was therefore abandoned.

(b) Addition of dibenamine to the nutrient bath. Dibenamine is known to produce an irreversible and unsurmountable blockade of the 5-HT stimulation, while leaving unaffected the uterine response to all other known interfering substances. If dibenamine blockade of the stimulant effect of brain extracts is complete, all stimulation may be reasonably ascribed to 5-HT. If it is incomplete, the stimulant effect can be attributed only in part to 5-HT; finally, if dibenamine is ineffective, the dominant presence of interfering substances may be inferred. Of course, the presence of interfering uterus-inhibiting compounds cannot be excluded by this procedure.

(c) Paper chromatography of the brain extract with subsequent elution of the area occupied by 5-HT, as indicated on control chromatograms by the colour test. It is a rather complicated procedure which has, however, the fundamental advantage that it rules out any interference by other active substances. Indeed, when using two different solvent systems, one acid and the other alkaline, there is practically no possibility that the 5-HT spot could contain other disturbing products, either stimulant or inhibiting. Since the loss of 5-HT in this technique is considerable, the actual content of 5-HT in brain extracts must be calculated from the recovery of added 5-HT.

RESULTS

Completeness of extraction of 5-HT *by different acetone concentrations.* In order to determine the completeness of extraction of *5-HT* from tissues by different volumes, i.e. by different concentrations of acetone, experiments were carried out not only on

mammalian brain but, for comparison, also on posterior salivary glands of *Octopus vulgaris*, which is a tissue extremely rich in 5-HT.

About 60 g of fresh posterior salivary glands were minced and thoroughly mixed. Three aliquots of 15 g each were treated, for 24-h periods, successively with the following solvents:

Aliquot *a*: 20 vol of pure acetone, 10 vol of 95% acetone, 5 vol of 50% acetone, 4 vol of distilled water;

Aliquot *b*: 10 vol of pure acetone, 10 vol of 90% acetone;

Aliquot *c*: 4 vol of pure acetone, 4 vol of 80% acetone, 4 vol of 50% acetone, 4 vol of distilled water.

The nine extracts were titrated separately.

Another sample of 80 g of fresh salivary glands was treated exactly as above. The results are shown in Table 1.

VOL. 1 56/57

TABLE 1.—EXTRACTION OF 5-HT FROM *Octopus* SALIVARY TISSUE BY DIFFERENT ACETONE CONCENTRATIONS (IN PARENTHESES)

	Solvent	5-HT (μg/g tissue)	Percentage extraction (172 μg = 100%)
Sample 1			
Aliquot *a*	20 vol pure acetone (95%)	64·5	37·5
	10 vol 95% acetone (95%)	21·5	12·5
	5 vol 50% acetone (50%)	51·5	30·0
	4 vol dist. water (0%)	23·5	13·7
		161·0	93·7
Aliquot *b*	10 vol pure acetone (90%) / 10 vol 90% acetone (90%)	138	80
Aliquot *c*	4 vol pure acetone (80%)	148·35	86·25
	4 vol 80% acetone (80%)	19·35	11·25
	4 vol dist. water (0%)	4·3	2·50
		172·0	100·0
			(258 μg = 100%)
Sample 2			
Aliquot *a*	20 vol pure acetone (95%)	38·7	15·0
	10 vol 95% acetone (95%)	17·0	6·6
	5 vol 50% acetone (50%)	90·3	35·0
		146·0	56·6
Aliquot *b*	4 vol pure acetone (80%)	219·3	85·0
	4 vol 80% acetone (80%)	31·4	12·0
	4 vol dist. water (0%)	7·3	3·0
		258·0	100·0

Calf brain (45 g) was minced and thoroughly mixed, then divided into three aliquots of 15 g each: the first was extracted twice with 4 vol of pure acetone and then with 4 vol of 80% acetone; the second was treated in the same manner, after 0·85 μg of 5-HT per gram tissue had been added; the third was extracted, after addition of the same quantity of 5-HT, with 20 vol of pure acetone and then with 10 vol of 95% acetone.

Rat, cat, and rabbit brain, as well as midbrain plus the diencephalon of a horse, were treated in a similar manner. Table 2 shows the extraction of tissue 5-HT and the recovery of added 5-HT for 95% (20 vol pure acetone/g tissue) and 80% acetone (4 vol pure acetone/g tissue).

TABLE 2.—EXTRACTION OF TISSUE 5-HT AND RECOVERY OF ADDED 5-HT BY 80% ACETONE (4 VOL PURE ACETONE/G TISSUE) AND 95% ACETONE (20 VOL PURE ACETONE/G TISSUE)

Tissue	Added 5-HT (μg/g tissue)	Acetone concentration (%)	5-HT content (μg/g tissue)	Percentage 5-HT recovery
Calf brain	0	80	0·32	—
Calf brain	0·85	80	1·16	99
Calf brain	0·85	95	0·43	37
Rat brain	0	80	0·40	—
Rat brain	0·40	80	0·76	95
Rat brain	0·40	95	0·18	22·5
Cat brain	0	80	0·20	—
Cat brain	0·80	95	0·16	16
Guinea-pig brain	0	80	0·28	—
Guinea-pig brain	0·80	80	1·0	93
Horse midbrain + diencephalon	0	80	0·3	—
Horse midbrain + diencephalon	1·25	80	1·5	97

The following conclusions may be drawn from Tables 1 and 2:

(1) A final acetone concentration of 80% permits a complete extraction of tissue 5-HT and a satisfactory recovery of added activity. This is in accordance with all the data of the literature.

(2) 95% acetone is inadequate for a satisfactory extraction of 5-HT from the posterior salivary glands of *Octopus* as well as from nervous tissue of mammals. Recovery of added 5-HT is exceedingly low (16–38%). With 90% acetone, about 80% of the 5-HT contained in the salivary tissue passes into the solvent.

(3) Minced salivary glands pretreated with 95% acetone retain their 5-HT more tenaciously than does fresh tissue, as shown by the incomplete extraction obtained with subsequent 50% acetone.

Recovery of added 5-HT on paper chromatograms of brain extracts. Paper chromatography was the procedure we preferred for determining the actual 5-HT content in

nervous tissue. As already stated, every application on paper of the brain extract alone was accompanied by a control application of the same quantity of the extract plus a known amount of 5-HT (0·2–2·0 μg per gram tissue). In twelve experiments with the butanol-acetic acid-water mixture, the average recovery of added 5-HT was 48% (range 33–61%); in fifteen experiments with the butanol-methylamine mixture the recovery was poorer (mean 30%; range 20–40%). From the percentage recovery of added 5-HT, a factor may be calculated by which the activity of the 5-HT spot given by the brain extract alone must be multiplied in order to obtain the actual content of 5-HT in the examined extract.

Activity of the brain extracts on uterine muscle. The 5-HT-equivalents of brain extracts obtained with 80% acetone as well as the effect of dibenamine on the uterus-stimulating action of these extracts are shown in Table 3.

As previously stated, not all the stimulating activity of brain extracts on uterine muscle can be considered attributable to 5-HT. In this connection, the interference of substance P seems to be particularly important.

Dibenamine allows a first orientation on the presence of disturbing uterus-stimulant substances. If all the stimulating activity of an extract is blocked by dibenamine, we consider that the 5-HT-equivalent of this extract does represent its actual content in 5-HT. The corresponding figures have been, consequently, transferred to Table 4. However, in the more frequent case when dibenamine blockade was incomplete, the actual 5-HT content of the extract was determined by paper chromatography followed by elution of the 5-HT spot (see Methods).

The 5-HT content of the examined brain extracts, as determined by the use of dibenamine and of paper chromatography, is shown in Table 4.

Paper chromatographic detection of 5-HT in brain extracts. This was tried with satisfactory results on extracts of pig mesencephalon plus diencephalon and of rat brain. An exact superimposition of the 5-HT spot given by the tissue extract and that given by added 5-HT was obtained with both the solvent systems used, and a perfect similarity of the colour reactions was observed. At an approximate estimation, the 5-HT content of the pig extract was 0·4 μg/g; that of the rat extract 0·3 μg/g. These values agree fairly well with those obtained by bioassay. The quantity of extract applied on paper corresponded to 0·5–1·0 g of tissue.

DISCUSSION

There is at present little doubt that the central nervous system of vertebrates contains a substance which is biologically and chromatographically indistinguishable from 5-HT.

In agreement with AMIN, CRAWFORD, and GADDUM (1954), it has been found in the present investigation that in most cases the tissues richest in 5-HT are the diencephalon and the midbrain. It is, however, doubtful whether this statement is valid for all animal species. Since exact information on the distribution of 5-HT in the central nervous system (so much the better if in comparison with that of substance P and sympathin) is important in many respects, it is desirable that the insufficient comparative data should be completed.

Once it has been agreed that 5-HT is a normal constituent of the nervous system, three fundamental problems are to be faced: that of the exact localization of 5-HT

TABLE 3.—5-HT-EQUIVALENTS OF BRAIN EXTRACTS OBTAINED WITH 80% ACETONE. THE NUMBERS IN PARENTHESES DENOTE THE NUMBER OF EXAMINED ANIMALS

Animal species	5-HT-equivalents (μg per gram fresh tissue)					
	Total brain		Basal ganglia + diencephalon + mesencephalon		Cortex	
	Mean	Range	Mean	Range	Mean	Range
Mammals						
Human foetus (1) (6 months)	—	—	0·05[a]	—	—	—
Cow (1)	—	—	0·10[a]	—	0·03[a]	—
Horse (2)	—	—	0·4[b]	—	0·05	—
Sheep (1)	—	—	—	—	0·07[b]	—
Hog (3)	—	—	0·57[a]	0·4–0·7	0·10[b]	—
Dog (5)	0·2[b]		0·80[b]	0·7–1·0		
Cat (4)	—	—	0·70[b]	0·5–0·8	0·40[a]	0·2–0·6
Rabbit (7)	—	—	0·3	—	0·03	—
Guinea-pig (5)	0·3[a]	0·2–0·5	—	—	—	—
Hedge-hog (2)	0·3[b]	—	—	—	—	—
Rat (30)	0·4[a]	0·3–0·6	—	—	—	—
Birds						
Pigeon (4)	0·25[b]	0·15–0·4	—	—	—	—
Hen (3)	0·27[b]	0·20–0·4	—	—	—	—
Turkey (1)	0·90[b]	—	—	—	—	—
Reptiles						
Lizard (10)	0·1[a]	—	—	—	—	—
Tortoise (4)	0·1[a]	—	—	—	—	—
Tropidonotus natrix (2)	0·2[a]	—	—	—	—	—
Amphibians						
Frog (40)	3–4[c]	—	—	—	—	—
Fishes						
Orthagoriscus mola (1)	0·2[c]	—	—	—	—	—
Tynnus tynnus (1)	0·2[c]	—	—	—	—	—
Scylliorhinus canicula (5)	0·4[b]	—	—	—	—	—

[a] Complete blockade of the uterine contractions by dibenamine.
[b] Partial blockade.
[c] No blockade.

within the nervous tissue, that of the relation existing between 5-HT in blood and gastro-intestinal tract and that in the central nervous system, and, finally, that of the biological significance of 5-HT in the cerebro-spinal axis.

(1) Two observations demonstrate that 5-HT may be localized in structures or cells other than true nerve cells:

(a) AMIN, CRAWFORD, and GADDUM (1954) have shown that, in the central nervous

VOL 1 1956/

system of the dog, one of the parts richest in 5-HT is the area postrema, which is a vascular area containing neuroglia but no true nervous tissue.

(b) In invertebrates 5-HT is contained in peripheral nerves in amounts similar to those present in ganglia.

TABLE 4.—ESTIMATES OF 5-HT (μg/g) IN THE NERVOUS SYSTEM OF VERTEBRATES

Animal species	Tissue		
	Total brain	Basal ganglia + diencephalon + mesencephalon	Cortex
Human foetus	—	0·05	?
Cow	—	0·1	0·03
Horse	—	0·3	0·03
Sheep	—	0·04	0·02
Hog	—	0·5	?
Dog	0·1	0·5	—
Cat	0·03	0·03	0·03
Rabbit	0·3	—	—
Guinea-pig	0·3	—	—
Rat	0·4	—	—
Pigeon	0·15		
Hen	0·2		
Turkey	0·4	—	—
Lizard	0·1	—	—
Tortoise	0·1	—	—
Tropidonotus natrix	0·2	—	—
Scylliorhinus canicula	0·2 (?)	—	—

The significance of these observations is clear, and it would certainly be an outstanding achievement to succeed in identifying exactly the site of production or storage of 5-HT in the nervous system.

The fact that WOOLLEY and MURRAY (1955) found that 5-HT causes oligodendroglial cells of human and rat brains cultured *in vitro* to contract strongly should not be ignored. As these cells normally pulsate and possibly serve as a device for causing the circulation of cerebral fluid other than blood, 5-HT could play a role in regulating the function of these cells rather than that of the true nerve cells.

(2) ERSPAMER (1955) is of the opinion that 5-HT in the nervous system is independent, both in regard to origin and to physiological significance, from gastrointestinal and blood 5-HT, which constitute approximately 99% of the 5-HT in the entire organism. It is a well-established fact that in invertebrates 5-HT may occur in nervous tissue, without being present in other tissues or hemolymph (cuttle-fish).

For vertebrates, a crucial experiment was tried in the present investigation. *Elasmobranchii* amongst the fishes are known to possess authentic enterochromaffin cells and enteramine in the gastro-intestinal mucosa; *Teleostei* are lacking in them. The problem was to investigate whether these two groups of fishes contained 5-HT in their central nervous system. If both groups contained the substance, the independence of brain 5-HT from blood and gastro-intestinal 5-HT would have definitely been proved; if, on the contrary, only *Teleostei* had been lacking 5-HT, a strict interdependence between the two localizations of 5-HT would have appeared probable.

Unfortunately, the scarcity of the available material (a tunny weighing approximately 100 kg yielded only 6 g of brain) and the unexpected presence in the brain extracts of high concentrations of other uterus-stimulating substances caused the failure of this first experiment. The problem will be reinvestigated.

(3) In 1953 GADDUM first postulated that "the 5-HT in our brain plays an essential part in keeping us sane," and the powerful effect of lysergic acid diethylamide on the human brain might be due to the inhibitory action of this drug on the 5-HT in the central nervous system. The concept of GADDUM and his collaborators was accepted and further developed by WOOLLEY and SHAW (1954), who formulated it as follows: (a) 5-HT probably plays a role in maintaining normal mental processes; (b) metabolically induced deficiency or excess of 5-HT in the brain may contribute to the production of some mental disorders; (c) 5-HT or a long-acting derivative of it capable of penetrating into the brain may prove useful in alleviating disorders similar to schizophrenia.

The most recent authoritative voice in favour of the theory that 5-HT has a role in brain function is that of BRODIE and collaborators (1955). Having observed that reserpine causes a release of 5-HT from the brain tissue, as it does from the gastro-intestinal tract, these investigators think that "reserpine action may be mediated through the liberation of serotonin in the brain" and, as a consequence, that 5-HT itself may be considered as a neurohormonal agent.

The above theories have been in part criticized by ERSPAMER (1955) and by ROTHLIN and CERLETTI (1955). Everyone, we think, will agree with AMIN, CRAWFORD, and GADDUM (1954) that "the 5-HT in the brain may also have some specialized role to play, the precise nature of which we do not yet know," but we believe that more binding conclusions do not seem justified by the limited available data.

In regard to BRODIE's theory, it is very difficult to conceive, as BRODIE and co-workers (1955) seem to do, that only the 1% of 5-HT which is contained in the brain has an important function, and the remaining 99%, which is in the gastro-intestinal mucosa, the blood platelets, and the spleen, must be considered as mere transport or depot 5-HT. Moreover, as BRODIE's theory is virtually based only on the fact that reserpine possesses contemporaneously a 5-HT-releasing activity and several actions on nervous and mental processes, it would be enough to discover a 5-HT-releasing drug which lacks central nervous effects to make the above theory untenable.

Something similar has already happened in the case of lysergic acid diethylamide. The discovery of 2-bromo-D-lysergic acid diethylamide (BOL-148) has shown that the peculiar mental effects of LSD were not due, as previously suggested, to an interference of this drug with 5-HT.

VOL
1
1956/

SUMMARY

In experiments carried out on posterior salivary glands of *Octopus vulgaris* and on brain tissue of mammals, 80% acetone appeared to be the most suitable solvent for the complete extraction of 5-HT from the tissues examined. With 95% acetone, the extraction of tissue 5-HT and the recovery of added 5-HT was unsatisfactory.

Dibenamine blockade and ascending paper chromatography were used in order to separate the 5-HT contained in brain extracts from other disturbing active substances.

The brain tissue of all mammals, birds, and reptiles studied contained detectable amounts of 5-HT. The areas richest in 5-HT were usually the diencephalon and the mesencephalon. The results of the bioassay could be confirmed by paper chromatography.

The presence of 5-HT in the brain of vertebrates as well as in nervous tissue of invertebrates is consistent with the theory that 5-HT may have some specialized role to play in the nervous tissue. The nature of this role is as yet obscure.

VOL.
1
56/57

REFERENCES

AMIN A. H., CRAWFORD T. B. B., and GADDUM J. H. (1954) *J. Physiol.* **126,** 596–618.

BRODIE B. B., PLETSCHER A., and SHORE P. A. (1955) *Science* **122,** 968.

CERLETTI A. and ROTHLIN E. (1955). *Nature* **176,** 785–786.

ERSPAMER V. (1954). *Rendiconti scient. Farmitalia* **1,** 1–193.

ERSPAMER V. (1955). *Medicina (Parma)* **5,** 1–34.

ERSPAMER V. (1956) Personal communication.

FLOREY E. and FLOREY E. (1953). *Naturwissenschaften* **40,** 413–414.

FLOREY E. and FLOREY E. (1954) Über die mogliche Bedeutung von Enteramin (5-Oxy-Tryptamin) als nervöse Aktionssubstanz bei Cephalopoden und dekapoden Crustaceen. *Z. Naturforschg.* **9b,** 58–68.

GADDUM J. H. (1954). *Ciba Foundation, Symposium on Hypertension.* J. and A. Churchill, London, pp. 75–77.

GARVEN J. D. (1955) Personal communication to Dr. V. Erspamer.

PERNOW B. (1955). In GADDUM'S *Polypeptides which stimulate plain muscle.* E. and S. Livingstone, Edinburgh and London, pp. 28–38.

TWAROG B. M. and PAGE I. H. (1953). *Amer. J. Physiol.* **175,** 157–161.

VOGT M. (1954). *J. Physiol.* **123,** 451–481.

WELSH J. H. (1954). *Federation Proc.*, **13,** 162–163.

WELSH J. H. (1955). *The Hormones*, vol. III, pp. 97–152. Academic Press Inc. New York.

WOOLLEY D. W. and SHAW E. (1954). *Brit. M. J.* **2,** 122–126.

WOOLLEY D. W. and MURRAY (1955) Personal communication.

Journal of Neurochemistry, 1956, Vol. 1, pp. 32–41, Pergamon Press Ltd., London.

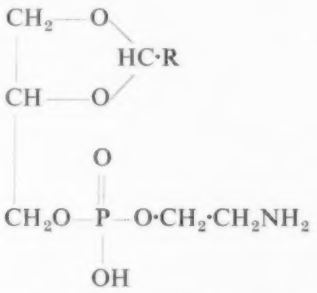
OBSERVATIONS ON THE ACETALPHOSPHOLIPIDS OF BRAIN TISSUE

G. B. ANSELL and JOYCE M. NORMAN
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(*Received* 17 *December* 1955)

INTRODUCTION

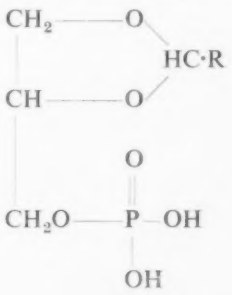
IN 1929 FEULGEN, IMHAUSER and BEHRENS demonstrated the presence of long-chain fatty aldehydes in muscle and brain tissue, and ten years later FEULGEN and BERSIN (1939) isolated from muscle tissue a lipid which they called acetalphospholipid and to which they ascribed the formula (I).



(I)

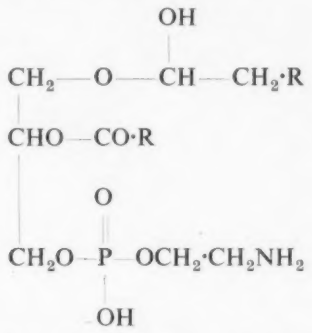
THANNHAUSER, BONCODDO, and SCHMIDT (1951a) isolated an acetalphospholipid in low yield from brain tissue. A lipid of the same type as (I) (2-aminoethyl 2 : 3-O-hexadecylidene-1-glycerophosphate) has recently been synthesized by EGERTON and MALKIN (1953).

It has generally been assumed that the acetalphospholipids are stable to alkali, and both FEULGEN and BERSIN (1939), and more recently KLENK (1944), have used hot alkali to destroy ester phosphatides in lipid mixtures and leave acetalphospholipids intact, although it is known that hot alkali treatment leads also to the formation of some plasmalogenic acid (acetalphosphatidic acid) (II) (FEULGEN and BERSIN, 1939). On the other hand, acetalphospholipids are generally considered to be very unstable to acids, which decompose them into the free aldehyde and glycerylphosphoryl-ethanolamine (GPE) (THANNHAUSER *et al.*, 1951b).



(II)

The fact, however, that unsaponified brain phospholipids yield GPE only slowly in the presence of acid (SCHMIDT, OTTENSTEIN, and BESSMAN, 1953; ANSELL and NORMAN, 1953) suggested that the native acetalphospholipids may exist in the tissues in a different form from (I). Very recently KLENK and DEBUCH (1954) have confirmed this, and on the basis of considerable experimental evidence have concluded that native acetalphospholipid is probably of type (III).



(III)

The present paper describes the separation of GPE from the ethanolamine-containing acetalphospholipid of brain tissue by the use of ion-exchange resins and paper chromatography. It is shown that the properties of the GPE so obtained differ from those of α-GPE expected from a compound of type (I), but they are consistent with its having derived from an acetalphospholipid of type (III). The properties of the acetalphospholipid isolated by THANNHAUSER *et al.* (1951b) from the saponified lipids of brain tissue were compared with those of the synthetic acetalphospholipid of EGERTON and MALKIN: it was shown that the acetalphospholipid isolated from brain tissue is unlikely to be a compound of type (I).

The presence of a serine-containing acetalphospholipid in brain tissue suggested by KLENK and BÖHM (1951) and AMELUNG and BÖHM (1954) was confirmed.

METHODS

Separation of the Water-soluble Constituents of Acetalphospholipids

(a) *Extraction and hydrolysis of rat-brain lipids*

This method is similar to that of SCHMIDT, BENOTTI, HERSHMAN, and THANNHAUSER (1946). Brain tissue was removed from the animal as soon as possible after decapitation, and the tissue (1 g) dispersed in a mechanical "homogenizer" with 30 ml ethanol/ether (3 : 1 *v*/*v*). The dispersion and solvent were heated under reflux for forty-five minutes, and the lipid solution decanted through a Whatman No. 40 filter paper. The tissue residue was re-extracted at the Bp. for 30 min with 30 ml $CHCl_3$/methanol (1 : 1*v*/*v*), the solvent being decanted as before and the extraction repeated. The combined extracts were taken to dryness under reduced pressure at a temperature below 40°, and the residue redissolved in $CHCl_3$/methanol (1 : 1 *v*/*v*) and filtered.

The almost colourless lipid solution was washed with 0·25 M aqueous $MgCl_2$, as described by DAWSON (1954a), and excess $MgCl_2$ removed by washing with water. The lipid solution, thus freed from water-soluble contaminants, was taken to dryness under reduced pressure, emulsified with 2 ml of N NaOH, and maintained at 37° for 40–48 h. After cooling to 0°, 2 ml of N HCl was added followed by 4 ml of 10% (*w*/*v*) trichloroacetic acid solution. The mixture was allowed to stand at room temperature for 2 h, and filtered through a Whatman No. 42 paper; the combined filtrate and aqueous washings were then extracted three times with an equal volume of ether to remove most of the trichloroacetic acid.

Without further dilution, the solution was passed through a column of Amberlite IR-120 (H^+ form, 10 cm long, 1 cm diameter) to remove Na^+, and the effluent plus washings passed through a column of Amberlite IR-4B (OH^- form, 10 cm long, 1 cm diameter) to remove anions; the pH of the resultant effluent and washings was *ca.* 7.

(b) *Paper chromatography.*

A volume of solution equivalent to 200 mg tissue was subjected to descending two-dimensional chromatography on Whatman No. 4 paper, using phenol/H_2O (4 : 1 *w*/*w*) as the first solvent and "collidine" (DENT, 1948) as the second. *tert*-Butanol/trichloroacetic acid/H_2O (DAWSON, 1954b) was sometimes used as an alternative second solvent. When spots were to be eluted for periodate oxidation, single-dimensional chromatography in phenol/H_2O was used.

Amino-compounds were located on chromatograms with 0·2% (*w*/*v*) ninhydrin in 95% (*v*/*v*) ethanol. Phosphorus compounds were either located by the method of HANES and ISHERWOOD (1949) or that of WADE and MORGAN (1953); the latter method has the advantage that the phosphorus compounds are chemically unchanged and can be eluted for further study. Neither method was, however, particularly effective after irrigation of chromatograms with "collidine," since large false positive areas were obtained.

(c) *Estimation of total P and periodate-labile P of compounds separated by paper chromatography*

Total P. This was determined by the method of ANSELL and DAWSON (1951).

Periodate-labile P. This was estimated as follows (cf. BURMASTER, 1946). The P-containing spot located by the method of WADE and MORGAN (1953) was cut into small pieces and pulped in a small centrifuge tube with 4 ml of water; the surrounding blank area of equal weight was treated similarly. After centrifugation, the supernatant was decanted into a test-tube and the paper extracted with two further (1·5 ml) volumes of water. The volume of the combined extracts was adjusted to 6·9 ml and 0·1 ml of 0·1 N H_2SO_4 and 0·5 ml of 0·1 M periodic acid added (a 2·14% (*w*/*v*) solution of sodium metaperiodate in 3 N H_2SO_4). After mixing the contents of the tube, the reaction was allowed to proceed at room temperature for 1 h and the excess periodate was then decomposed with 1 ml of 4% (*w*/*v*) Na_2SO_3 solution. The resultant clear solution was made N with respect to H_2SO_4, heated in a boiling-water bath for 1 h, cooled, and diluted to 10 ml. The inorganic phosphate liberated was estimated by adding 1·0 ml of 12·5% (*w*/*v*) ammonium molybdate, and proceeding as described by BERENBLUM and CHAIN (1938). After development of the colour with $SnCl_2$, the *iso*butanol layer was washed with a further 10 ml of N H_2SO_4. Whatman No. 4 paper yielded a negligible blank under these conditions and 1 μg P could easily be estimated by the procedure.

EXPERIMENTAL RESULTS

Products of prolonged alkaline hydrolysis of brain lipids

When a solution of the water-soluble phosphorous compounds, produced by the prolonged saponification of brain lipid, was treated first with IR-120 (H^+ form) and then with IR-4B (OH^- form) resin, only two phosphorus compounds could be detected by paper chromatography; both of these also reacted with ninhydrin. The ion-exchange resins had removed the sodium chloride produced by the neutralization of the saponification mixture, and the large amounts of glycerophosphate liberated from the ester monoaminophosphatides.

One of the ninhydrin-reacting phosphorus compounds had an R_F of 0·67 in phenol/H_2O, and could be identified as GPE by methods previously described (ANSELL and NORMAN, 1953). It was shown further that, when an eluate of a spot, located by the method of WADE and MORGAN (1953), was hydrolysed with N hydrochloric acid at 100° for 1 h, the only phosphorus-containing substance which could then be detected on chromatograms was glycerophosphate.

VOL 1 1956/

The other substance had an R_F of 0·12 in phenol/H_2O and was obtained in much smaller amounts than GPE. The following experiments show that it had the properties of glycerylphosphorylserine (GPS):

(a) Its position on chromatograms was the same as that described for GPS by DAWSON (1954b).
(b) On acid hydrolysis it yielded only serine and glycerophosphate.
(c) A substance identical in properties was obtained when a preparation of serine kephalin (a mixture of ester- and acetalphospholipid, prepared by the method of FOLCH (1942) and kindly supplied by Prof. E. KLENK) was hydrolysed as described in the Experimental Section.

Since prolonged saponification causes the complete break-down of ester-phosphatides to fatty acids, the free base and glycerophosphate, the GPE and GPS, obtained under the conditions of the present experiments, must derive from acetalphospholipids. The fact that no other phosphate esters were obtained on the final chromatograms indicated that the other phosphate esters obtained by DAWSON (1954b) after brief alkaline hydrolysis for 15 min at 37° had either been further degraded or retained by the resins used in the separation. According to BAER and KATES (1950), a synthetic lecithin (phosphatidyl choline) is hydrolysed by 0·2 N sodium hydroxide to glycerylphosphorylcholine (GPC) and fatty acids in less than 1 h at 37°; but the GPC is known to be further hydrolysed to glycerophosphate and choline in 7 h at 37° by N sodium hydroxide (BAER and KATES, 1948).

The presence of free serine and glycine on the chromatograms was consistently observed, and no other amino acids were present. The serine may be assumed to derive from the phosphatidyl serine or serine acetalphospholipid, but the presence of glycine suggests that a glycine-containing lipid may be present. The association of amino acids with phospholipids has been described many times (see, for example, HECHT and MINK, 1952), but it appeared unlikely that the glycine found in the present experiments could have been uncombined. The existence of distinct lipopeptides (which might yield glycine on hydrolysis) has recently been denied (BODE and LUDWIG, 1954).

In Table 1 are given figures for the total acid- and alkali-labile phosphorus together with the GPE and GPS after removal of the glycerophosphate with the Amberlite resins. That GPE and GPS phosphorus together fall short of the total may be ascribed to losses during the paper chromatography. In the case of GPS there was a significant loss between irrigation in phenol and irrigation in collidine. The results indicate, however, that the amount of GPS (and hence the amount of serine acetalphospholipid) in rat-brain tissue is approximately 10% of the ethanolamine analogue. A sample of ethanolamine kephalin, kindly provided by Prof. KLENK, which had a phosphorus content of 3·5% and a fatty-aldehyde content of 17% (calculated as the dimethylacetal of a mixture of 50% palmitaldehyde and 50% stearaldehyde) yielded GPE equivalent to 34% of the total lipid phosphorus. The acetalphospholipid-phosphorus content calculated from the aldehyde content is equivalent to 49% of the total lipid phosphorus.

Constitution of the ethanolamine acetalphospholipid of brain

(a) *Experiments with the GPE from brain acetalphospholipid.* In a number of experiments, the GPE separated by single-dimensional chromatography was eluted with water and oxidized with periodic acid, as described in the Experimental Section

TABLE 1.—WATER-SOLUBLE PHOSPHATE ESTERS DERIVING FROM THE ACETALPHOSPHOLIPIDS OF RAT-BRAIN

Rat No.	μg P/g fresh brain tissue			
	Total acid- and alkali-labile P	P after treatment with Amberlite IR-120 (H^+) and IR-4B (OH^-)		
		Total	GPS	GPE
1	1505	314	4·5	160
2	1710	340	13·4	—
3	—	260	15·1	236
4	1615	276	19·1	150
5	1870	272	7·2	148
6	1995	338	—	213
7	1770	146	24·0	122
8	1630	146	7·5	85
Mean	1728	262	13·0	155

of this paper. Such an oxidation procedure is known to liberate all the phosphorus of α-glycerophosphate, and a sample of L-α-GPE (synthesized by Prof. BAER and kindly supplied by Dr. R. M. C. DAWSON) was shown to behave similarly. However, the figures given in Table 2 for the acetalphospholipid-GPE from rat-brain demonstrate that only half behaves as the α compound.

TABLE 2.—THE LABILITY OF ACETALPHOSPHOLIPID GPE TO PERIODIC ACID

Expt. No.	Source	Total GPE (μg P)	μg P labile to to HIO_4 at 15°	μg P labile to HIO_4 at 100° in presence of NH_2SO_4	% α
1	Rat brain	212·6	120·6	137	57
2	Rat brain	122·5	58·6	51·1	48
3	Rat brain	84·5	42	(63·1)	50
4	Rat brain	236	121	—	51
5	Rat brain	206	140	—	68
6	Ethanolamine kephalin	12·2	6·1	5·1	50
				Mean %	54%

The following experiments give evidence that the remainder of the GPE is in the β form:

(i) The eluate was heated with 0·135 M periodic acid in the presence of N sulphuric acid at 100° for 1 h, a procedure which liberates all the phosphorus of α- and β-glycerophosphate as inorganic phosphate (BURMASTER, 1946). The amount of inorganic phosphate liberated at 100° from the GPE was not significantly greater than that liberated at 15° (Table 2).

(ii) The GPE moved as a single spot in all the chromatographic solvents examined, including

phenol/water (4 : 1 *w*/*w*), "collidine" (DENT, 1948) and *n*-propanol/ammonia/water (HANES and ISHERWOOD, 1949).

(iii) Hydrolysis of the GPE eluate with 5 N hydrochloric acid at 100° for 16 h rendered all the phosphorus labile to periodic acid at room temperature.

(iv) Acid hydrolysis of the GPE eluate led to the exclusive production of ethanolamine and glycerophosphate.

(v) When a sample of ethanolamine kephalin (a mixture of ester- and acetalphospholipid prepared from brain tissue by the Folch (1942) procedure and kindly supplied by Prof. KLENK) was subjected to the saponification procedure, approximately half of the GPE so obtained was also resistant to hot periodic acid (Table 2).

(vi) A sample of a synthetic β-phosphatidyl ethanolamine (kindly supplied by Dr. MALKIN) was catalytically hydrolysed with mercuric chloride, and the GPE separated by single-dimensional chromatography in phenol/water (NORMAN and DAWSON, 1953). This GPE, which could not have been other than a mixture of α and β forms, was also partly resistant to hot periodic acid.

It appears certain from the foregoing evidence that the GPE deriving from rat-brain acetalphospholipid *after* saponification is a mixture of the α and β form. The implications of such a finding are discussed below.

(b) *Experiments on* 2-*aminoethyl* 2:3-O-*hexadecylidine*-1-*glycerophosphate*. The following experiments were carried out on the synthetic acetalphospholipid kindly supplied by Dr. MALKIN. It had a phosphorus content of 7·16% (theory = 7·10%) after drying at 85° for 30 min.

(i) The lipid (1·73 mg) was treated with 0·25 ml of N sodium hydroxide solution, in which it was completely soluble, and maintained at 37° for 48 h. After dilution with water, the solution was neutralized with hydrochloric acid and an equal volume of 10% (*w*/*v*) trichloroacetic acid added. After 2 h at room temperature, the mixture was filtered and the acid-soluble phosphorus determined (cf. SCHMIDT *et al.*, 1946). No acid-soluble phosphorus was obtained (Table 3).

TABLE 3.—EFFECT OF VARIOUS HYDROLYSIS PROCEDURES ON 2-AMINOETHYL-2:3-O-HEXADECYLIDENE-1-GLYCEROPHOSPHATE

Treatment	Acid-soluble phosphorus (as % of lipid phosphorus)	GPE-phosphorus* (as % of lipid phosphorus)	α-GPE-phosphorus (as % of GPE phosphorus)
(i)	0	—	—
(ii)	51	49	59
(iii)	—	81	68
(iv) a	56	32	43
(iv) b	42	30	66
(v) a	—	51	82
(v) b	75	63	68

* These values are minimal and do not take into account losses on the resin columns or chromatography.

(ii) The lipid (0·97 mg))was incubated with sodium hydroxide and neutralized with hydrochloric acid, as in (i). The mixture was then incubated with 3·3 N acetic acid at 37° for 48 h (cf. THANNHAUSER *et al.*, 1951b). The aqueous phase was passed first through a column of Amberlite IR-120 (H^+ form) and then through a column of Amberlite IR-4B (OH^- form). GPE was separated by single-dimensional paper chromatography of the effluent in phenol/water. Approximately half the available phosphorus was liberated by this treatment, and all was in the form of GPE (Table 3).

(iii) The lipid (0·97 mg) was catalytically hydrolysed with 0·02 N mercuric chloride for 3 h at 100° (NORMAN and DAWSON, 1953). After removal of the mercuric chloride with silver oxide and

hydrogen sulphide, GPE was separated as in (ii). At least 80% of the available phosphorus was converted to GPE by this procedure and half of it was in the β form (Table 3).

(iv) The lipid—(a) 1·13 mg, (b) 0·80 mg—was dissolved in 1 ml of glacial acetic acid and heated for 5 min at 55° with 1 ml of 2 N hydrochloric acid. After extraction with ether, the aqueous phase was diluted with water and passed over a column of Amberlite IR-4B (OH^- form); GPE was separated from the eluate, as in (ii). Only 30% of the available phosphorus was obtained as GPE by this procedure (Table 3) and approximately half of this was in the α form, but a further 12–26% was retained by the resin and could be eluted with N ammonium hydroxide; this was assumed to be glycerophosphate.

(v) The lipid—(a) 0·69 mg, (b) 0·89 mg—was hydrolysed in 2 ml of N methanolic hydrochloric acid for 5 min at 55° and, after extraction with ether, GPE was separated from the aqueous phase, as in (iv). Between 50–60% of the available phosphorus was obtained as GPE, and the yield of the α form was high (Table 3). A further 12% of the available phosphorus was retained by the resin and was assumed to be glycerophosphate.

These findings are discussed below.

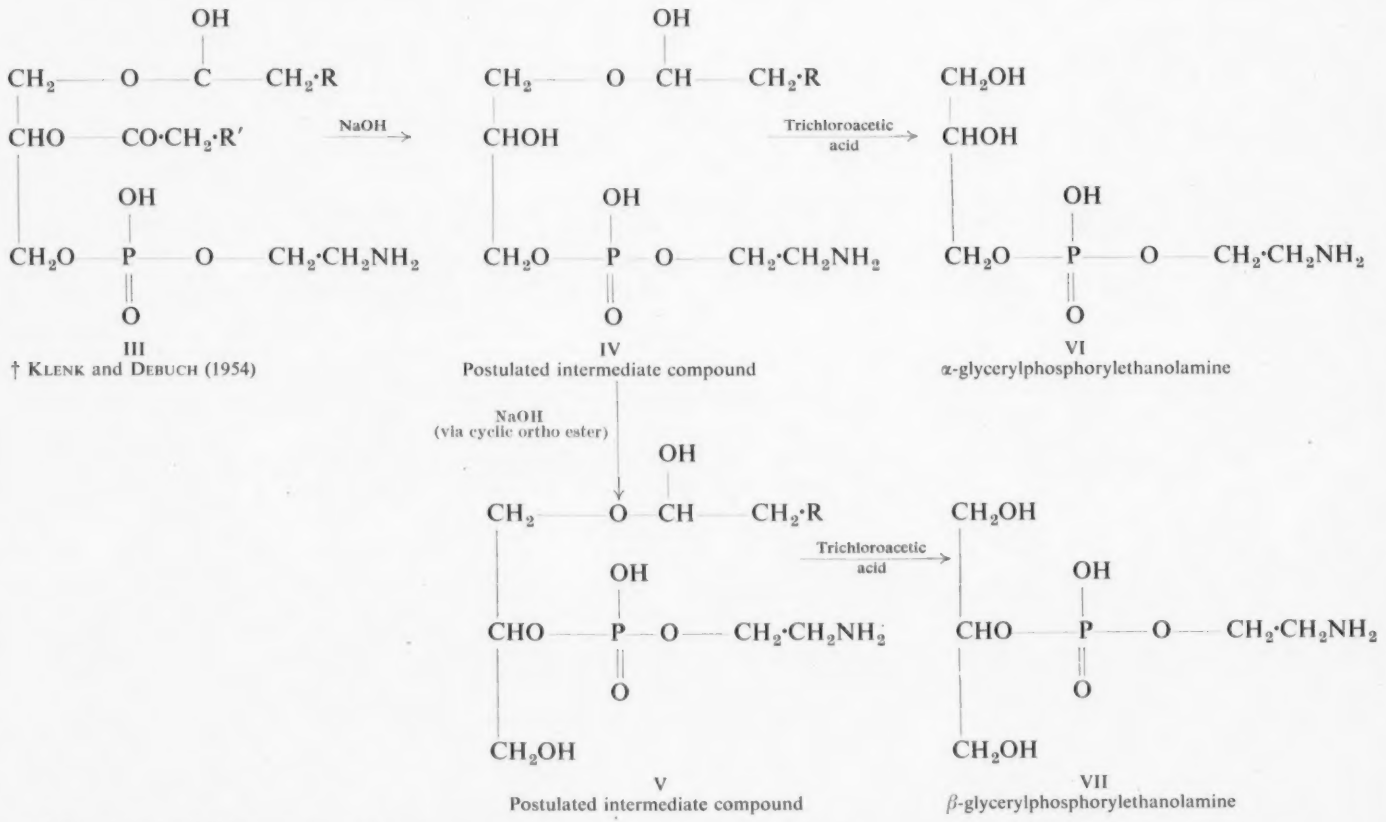
DISCUSSION

Although the existence of large amounts of acetalphospholipids (plasmalogens) in the tissues has been known for some time, neither the range of compounds involved nor their role in cell-function is yet known. Until recently it was thought that ethanolamine was the only organic base in the acetalphospholipids, but KLENK and BÖHM (1951) demonstrated the presence of fatty aldehydes in the serine kephalin fraction of brain tissue. A fuchsin-reacting substance inseparable from the serine kephalin fraction was also demonstrated by AMELUNG and BÖHM (1954), using paper chromatography. Since the presence of a choline-containing acetalphospholipid in heart-muscle, suggested by HACK (1953), has been recently established by KLENK and GEHRMAN (1953), it would appear that the diversity of the nitrogenous components of classical ester phospholipids is reflected also in the acetalphospholipids.

The fact that a mixture of α- and β-glycerylphosphorylethanolamine was consistently obtained by the hydrolysis of the brain acetalphospholipid raises an interesting point about the structure of the intact native lipid. It is clear that if the native lipid were of type (I), it should be resistant to mild alkaline hydrolysis (except for the possible release of a small amount of ethanolamine), and the subsequent treatment with weak acid would yield exclusively α-glycerylphosphorylethanolamine. THANNHAUSER *et al.* (1951b) found this to be true for the substance they isolated after saponification of brain lipids. The present results can, however, be explained on the basis of the formula (III), recently suggested for the native ethanolamine acetalphospholipid by KLENK and DEBUCH (1954), since a substance of this type would be partially labile to alkali with the formation of intermediates which could give rise to α- and β-glycerylphosphorylethanolamine on mild treatment with acid (Fig. 1).

The action of alkali on (III) would be to hydrolyse the fatty acid-ester linkage and yield an intermediate substance containing a free hydroxyl group in the α position (IV). Cyclic ortho-ester formation could then take place with the free hydroxyl group of the phosphate moiety, which could then yield approximately equal amounts of an α and β compound (IV) and (V). In the presence of acid a mixture of α- and β-glycerylphosphorylethanolamine (VI) and (VII) would result. This process would be analogous to the alkaline hydrolysis of α-kephalins and of α-lecithins, which yield a mixture of α- and β-glycerophosphoric acid (BAER, STANCER and KORMAN, 1953; BAER and KATES, 1950).

VOL 1 1956/



III
† KLENK and DEBUCH (1954)

IV
Postulated intermediate compound

VI
α-glycerylphosphorylethanolamine

V
Postulated intermediate compound

VII
β-glycerylphosphorylethanolamine

† Two other possibilities were suggested by these workers, but this scheme would not be affected.

If the structure of the native acetalphospholipid is as shown in (III), then treatment with acid should yield a lysokephalin. The formation of such a compound has not hitherto been demonstrated, as far as the authors are aware, although KLENK and DEBUCH (1955) have obtained a lysolecithin from the choline-containing acetalphospholipid of heart-muscle.

It would appear that the acetalphospholipid isolated by THANNHAUSER *et al.* (1951a), and assigned to the structure (I) by them has not this structure, but it is an intermediate compound formed by the saponification of the native acetalphospholipid. In agreement with this, experiments carried out on the synthetic acetalphospholipid of EGERTON and MALKIN (1953), which is a compound of structure (I), showed it to possess quite different properties from the acetalphospholipid of THANNHAUSER *et al.* The synthetic acetalphospholipid did not yield any acid-soluble phosphorus after saponification and acidification by the standard procedure of SCHMIDT *et al.* (1946), whereas all the phosphorus of native acetalphospholipid, as of any saponification product, is rendered acid-labile by this procedure. Only prolonged acid treatment of the saponified lipid yielded glycerylphosphorylethanolamine, and the yield was only 49% of the available phosphorus.

Catalytic hydrolysis of the lipid with mercuric chloride yielded at least 81% of the available phosphorus as glycerylphosphorylethanolamine, but only 68% of this was in the α-form: the substance isolated by THANNHAUSER *et al.* (1951b) yielded only α-glycerylphosphorylethanolamine by this procedure. When the lipid was hydrolysed in N hydrochloric acid for a very short period of time, only 40-75% of the available phosphorus was obtained as acid-soluble phosphorus. All the aldehyde of native acetalphospholipid is liberated by this procedure (FEULGEN, BOGUTH, and ANDRESEN, 1951), so that this result is a further indication that this lipid does not contain an αβ-diacetal linkage.

SUMMARY

(1) The separation of water-soluble phosphate esters from the acetalphospholipids of brain tissue is described.

(2) The amount of ethanolamine acetalphospholipid in rat-brain is approximately ten times greater than that of the serine analogue.

(3) The structure of the ethanolamine acetalphospholipid of brain is discussed in the light of the properties of the glycerylphosphorylethanolamine obtained from it and those of synthetic 2-aminoethyl 2:3-O-hexadecylidene-1-glycerophosphate.

Acknowledgements—We wish to thank Dr. D. RICHTER for his continuous encouragement whilst this work was in progress. The technical assistance of Miss ANNE PHILLIPS is also gratefully acknowledged. One of us (J. M. N.) is indebted to the Rockefeller Foundation for a personal grant, and we thank the Medical Research Council for a grant for expenses.

REFERENCES

AMELUNG D. and BÖHM P. (1954) *Hoppe-Seyl. Z.* **298,** 199.
ANSELL G. B. and DAWSON R. M. C. (1951) *Biochem. J.* **50,** 241.
ANSELL G. B. and NORMAN J. M. (1953) *Biochem. J.* **55,** 768.
BAER E. and KATES M. (1948) *J. biol. Chem.* **175,** 79.
BAER E. and KATES M. (1950) *J. biol. Chem.* **185,** 615.
BAER E., STANCER H. C., and KORMAN I. A. (1953) *J. biol. Chem.* **200,** 251.

BERENBLUM I. and CHAIN E. (1938) *Biochem. J.* **32,** 295.
BODE F. and LUDWIG U. M. (1954) *Klin. Woch.* **32,** 1097.
BURMASTER C. F. (1946) *J. biol. Chem.* **164,** 233.
DAWSON R. M. C. (1954a) *Biochem. J.* **57,** 237.
DAWSON R. M. C. (1954b) *Biochim. biophys. Acta.* **14,** 374.
DENT C. E. (1948) *Biochem. J.* **43,** 169.
EGERTON M. J. and MALKIN T. (1953) *J. chem. Soc.* 2800.
FEULGEN R. and BERSIN TH. (1939) *Hoppe-Seyl. Z.* **260,** 217.
FEULGEN R., BOGUTH W., and ANDRESEN G. (1951) *Hoppe-Seyl. Z.* **287,** 90.
FEULGEN R., IMHAUSER K., and BEHRENS M. (1929) *Hoppe-Seyl. Z.* **180,** 161.
FOLCH J. (1942) *J. biol. Chem.* **146,** 35.
HACK M. H. (1953) *Biochem. J.* **54,** 602.
HANES C. S. and ISHERWOOD F. A. (1949) *Nature* **164,** 1107.
HECHT E. and MINK C. (1952) *Biochim. biophys. Acta.* **8,** 641.
KLENK E. (1944) *Hoppe-Seyl. Z.* **281,** 25.
KLENK E. and BÖHM P. (1951) *Hoppe-Seyl. Z.* **288,** 98.
KLENK E. and DEBUCH H. (1954) *Hoppe-Seyl. Z.* **296,** 179.
KLENK E. and DEBUCH H. (1955) *Hoppe-Seyl. Z.* **299,** 66.
KLENK E. and GEHRMAN G. (1953) *Hoppe-Seyl. Z.* **292,** 110.
NORMAN J. M. and DAWSON R. M. C. (1953) *Biochem. J.* **54,** 396.
SCHMIDT G., BENOTTI J., HERSHMAN B., and THANNHAUSER S. J. (1946) *J. biol. Chem.* **166,** 505.
SCHMIDT G., OTTENSTEIN B., and BESSMAN M. J. (1953) *Fed. Proc.* **12,** 265.
THANNHAUSER S. J., BONCODDO N. F., and SCHMIDT G. (1951a) *J. biol. Chem.* **188,** 417.
THANNHAUSER S. J., BONCODDO N. F., and SCHMIDT G. (1951b) *J. biol. Chem.* **188,** 423.
WADE H. E. and MORGAN D. M. (1953) *Nature* **171,** 529.

VOL.
1
56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 42-53, Pergamon Press Ltd., London

THE QUANTITATIVE ESTIMATION OF CEREBROSIDES IN NERVOUS TISSUE

LARS SVENNERHOLM
Department of Medical Biochemistry, University of Gothenburg, Sweden

(*Received* 16 *December* 1955)

THE extensive research work in the physiological and pathological processes in which lipids are involved has increased the necessity for accurate micromethods for their quantitative estimation. BRANTE (1949) reviewed modern micromethods for the determination of the lipids in nervous tissue, and made a thorough investigation of the various factors which may influence them. Like most other investigators, BRANTE determined the cerebroside content only by estimating the reducing substances in a lipid extract before and after hydrolysis. From the important work of KLENK and collaborators (KLENK, 1941, 1942; KLENK and LANGERBEINS, 1941; SCHUWIRTH, 1940) we know that cerebrosides are not the only lipids containing carbohydrates in the central nervous system. They have isolated gangliosides from the brain and determined their amount in different nervous tissues. Besides these two glycolipids, a third has been described by ARSOVE, FOLCH, and MEATH (1951a). They named the new lipid strandin, but my preparations like DAUN'S (1952), showed that strandin consisted of gangliosides in a different physico-chemical state contaminated with low-molecular substances and mucopolysaccharides. CHATAGNON and CHATAGNON (1953, 1954) have suggested that sphingomyelin is also a part of the complex. BRANTE (1949) discussed the interference of gangliosides on the cerebroside values, and we (BRANTE and SVENNERHOLM, 1949, 1951) determined both total glycolipids and gangliosides in some foetuses. Other investigators (JOHNSON, MCNABB, and ROSSITER, 1950; CUMINGS, 1953; BLACKWOOD and CUMINGS, 1954) have neglected the gangliosides. Therefore the values for cerebrosides found in the current literature include gangliosides. EDGAR (1955) who has thoroughly discussed the problem, used the term glyco-sphingosides instead of cerebrosides. He has also made some estimations of both gangliosides and cerebrosides.

Consequently, the carbohydrates in a lipid extract are derived from at least two lipid sources. But lipid extracts are contaminated to some extent with low-molecular substances, partly of carbohydrate nature. It is impossible to remove them completely by the methods generally used, i.e., precipitation of the lipids before extraction or re-extraction of a primary lipid extract. Generally, the error introduced by the contaminants is negligible in the determination of glycolipids in adult nervous tissue, but this is not so in foetal tissue, where the amount of glycolipids is low and that of the contaminants is both relatively and absolutely higher. FOLCH, ASCOLI, LEES, MEATH, and LEBARON (1951b), however, succeeded in removing these substances by partition dialysis. By this procedure the gangliosides too are separated from the other lipids. It therefore appeared that it might be possible to use this method to separate cerebrosides from other substances containing carbohydrates.

VOL
1
1956/

The estimation of hexoses in cerebrosides can be done by two different methods:

1. Determination of the reducing power in alkaline solution after hydrolysis.
2. Determination of furfural formed in strong sulphuric acid.

1. The first method has been used almost exclusively, and many modifications of the original procedure (NOLL, 1899) have been made. The most convenient method appears to be that of BRAND and SPERRY (1941). The hydrolysis conditions are well established, and correction is made for reduction before hydrolysis. The released sugar reduces ferricyanide, and then an oxidimetric titration with cerium sulphate according to MILLER and VAN SLYKE (1936) is performed. The same method was used by BRANTE (1949). A disadvantage is that the consumption of ferricyanide by glucose and galactose is rather different. The reducing value of chondrosamine and sialic acid (neuraminic acid) in gangliosides (SVENNERHOLM, 1955c) has not been tested. Like all reduction methods, it requires relatively large amounts of material, at least 0·1 mg hexose in the sample.

2. In the other type of method the sugars are converted to furfural derivatives, which are then coupled to a phenol. The colour developed is determined colorimetrically. EDMAN (1942) used the carbazole method of DISCHE (1930), and BRÜCKNER (1941) modified the orcinol method of SÖRENSEN and HAUGAARD (1933). One advantage of this method is that it does not require so much material and is more specific. Hexosamine gives no reaction, and sialic acid can be compensated for. However, before hydrolysis, it is necessary to remove all carbohydrates other than those in the lipids.

Since it seemed that the second type of method was to be preferred, and as only incomplete tests of these methods for lipids have been made, this investigation was started.

MATERIALS AND METHODS

Reagents and Apparatus

Orcinol reagent—Orcinol (0·2 g), recrystallized twice from benzene, was dissolved in 100 ml analytical-grade H_2SO_4 (sp. gr. 1·84, 95–97%, MERCK). This keeps at least fourteen days when stored in a brown bottle in the refrigerator.

Blank reagent—The same sulphuric acid as used for the reagent.

0·1% *Saline*—Sodium chloride A.R. (100 mg) dissolved in 100 ml distilled water.

Methanol—Technical-grade methanol was dried above anhydrous K_2CO_3 and redistilled twice.

Chloroform—Technical-grade chloroform was dried over anhydrous $CaCl_2$ and K_2CO_3 and redistilled twice.

Carbohydrates—All sugars used were analytical reagents or C.P.-grade reagents. They were recrystallized from ethanol (galactose recrystallized twice). They were dried to constant weight in a vacuum desiccator over P_2O_5 and NaOH pellets. Stock solutions containing 1 mg/ml were prepared.

Cerebrosides—These were prepared according to the method of KLENK and LEUPOLD (1944). They contained no phosphorus.

Filter papers—The filter papers used (Munktell 00) were boiled with ethanol-ether (3 : 1 *v*/*v*) twice, and when used in the filtration of the hydrolysed lipid samples they were washed with 3 N sulphuric acid.

Microsoxhlet apparatus for heat extraction.

Thunberg tubes—Tubes 18 × 200 mm with ground stoppers and bore for evacuation.

Test-tubes—Pyrex tubes 15 × 160 mm.

Stirrers—The type used was described by VASSEUR (1948) (a piece of 5 mm glass tubing with a bulb of about 12 mm blown at one end). The stirrers and the tubes were boiled in chromic acid before use. When clean and dry, they were stored in closed polyethene boxes.

Glass-stoppered cylinders of 10 *and* 25 *ml*—The glass stoppers must fit exactly. They were moistened with water before use.

Water bath at 80°C. A "superthermostat" manufactured by Mr. E. Bühler, Tübingen, was used.

Tissues

Human material was used throughout. The foetal tissues were obtained immediately after operation (legal abortion), but the autopsy material was stored for 12–36 h after death in a cold room. The nervous tissue was homogenized either by the method of BRANTE (1949)—sectioning the

frozen tissue on a microtome table in 10 mμ-thick sections—or that of SPERRY (1954). The homogenized tissue was dried by lyophilization or in a vacuum desiccator over H_2SO_4 and NaOH pellets. After this primary drying, the tissue was ground to a powder in an agate mortar and dried again, this time over P_2O_5 and NaOH, to constant weight.

Extraction Procedure

The extraction of the dried tissue was performed in the microsoxhlet apparatus for two hours with a solvent mixture of 2 parts methanol and 1 part chloroform (by volume). The extract was filtered through a small filter paper (5·5 cm diam.) into a 25 ml cylinder, and solvent was added to the 10ml mark. The flask and filter paper were rinsed with 10 ml chloroform, and then 0·1% saline was added to 25 ml. The cylinder was shaken vigorously for 1 min and left for 48 h. The upper phase was removed with a balloon pipette and the lower phase washed three times with 3 ml of the upper phase from a mixture of 800 ml chloroform-methanol (2 : 1 *v/v*) and 200 ml 0·1% saline. The lower phase was taken to the 25 ml mark with chloroform-methanol (2 : 1 *v/v*).

Cerebroside Estimation

An aliquot of the lipid extract was transferred to a Thunberg tube and evaporated in a stream of nitrogen at 50°. The residue was dissolved in 0·5 ml ethanol, and 2 ml 3 N H_2SO_4 was added. The hydrolysis was performed in the closed tube for 2 h in a boiling-water bath. After cooling in running water, the hydrolysate was filtered into a 10 ml cylinder. The hydrolysis tube was rinsed three times with 1 ml distilled water, which was then transferred to the filter. Afterwards, distilled water was added to the mark.

Three samples of 2 ml hydrolysate were pipetted into the test-tubes. The rack with the test-tubes was put in the ice-bath. After 15 min, 4 ml orcinol reagent was added to two of the samples. To the third, 4 ml blank reagent was added instead. After 15 min, the solutions were thoroughly mixed with the stirrers, and then the rack was placed in the water bath at 80° for exactly 20 min. After heating, the tubes were chilled in the ice bath and then read in Beckman spectrophotometer B at 505 mμ in cuvettes with 1·00 cm light path.

In each run, standards of 0, 50, 100, and 200 μg galactose were run through all the steps. The content of galactose was estimated from the standard curve after subtraction of the sample blank. The cerebroside content was calculated by multiplying the galactose value by 4·6.

RESULTS

The procedure described was designed to allow the determination of gangliosides in the upper phase (SVENNERHOLM, 1955a). The drying and homogenizing methods were tested in ganglioside determinations, and no differences were found between the two homogenizing and the drying methods. It was, however, very important to have a really dry and finely pulverized material.

Extraction

FOLCH *et al.* (1951b) have described an extraction procedure in which the lipids are extracted from fresh nervous tissue with chloroform-methanol (2 : 1 *v/v*), and the contaminating substances are removed by partition dialysis. However, together with the lipids, a mucopolysaccharide containing hexosamine, hexose, and sialic acid (SVENNERHOLM and SVENNERHOLM, to be published) is extracted. Owing to this, extracts prepared according to FOLCH *et al.* (1951b) cannot be used if gangliosides are also to be analysed. The partition between the two phases will also be influenced by the mucopolysaccharides, which delay the separation. A method was therefore chosen in which the lipids were extracted from dried material. In order to reduce the uptake of water during extraction, a sand bath supplied the heat.

A sample of 10–60 mg was weighed and placed on a filter paper (40 × 40 mm), which was folded so that the substance was well enclosed and then placed in the

extraction apparatus. The extraction was performed with 10 ml methanol-chloroform (2 : 1 *v/v*), which gave the optimal amount of glycolipids (different mixtures of methanol-chloroform from 4 : 1 to 1 : 2 were investigated). Two hours were necessary for complete extraction under the conditions used.

Even though great care was observed in the folding of the filter paper, small amounts of solid substance sometimes went into the extraction flask. The extract was therefore filtered into the 25 ml cylinder through a hard filter. HACK (1952) has reported that phospholipids may be adsorbed on filter paper, but no such effect was observed with the glycolipids.

Removal of Contaminating Substances

The lipid extract had to be freed from gangliosides and non-lipid carbohydrates. For two years the method of FOLCH *et al.* (1951b) was used. Inside a 1000 ml beaker was placed a small one, and the big beaker was filled with distilled water so that the upper edge of the small beaker was at least 3 cm beneath the water surface. An aliquot of a lipid extract of methanol-chloroform (1 : 2 *v/v*) was then placed in the small beaker and left for 72 h. It was technically difficult to add the lipid extract in that way, and the method was modified. The lipid extract was first placed in the small beaker, and the distilled water was then carefully added. The same modification has also been described by SPERRY (1954). Duplicate determinations of lipid hexose generally showed good agreement, but completely different results were often obtained without it being possible to find any explanation.

In the method finally adopted the partition was carried out in 25 ml cylinders. The proportions of methanol and chloroform were the same as those used by FOLCH *et al.* (1951b); to four parts of solvent mixture one part of salt solution was added. The optimal concentration of the saline was determined by analysing lipids in the water phase and gangliosides in the chloroform phase. When distilled water only was tried, no distinct separation occurred. The lowest concentration of saline which gave an optimal partition was 0·1 %. In that case no lipids other than gangliosides were found in the water phase. The amount of gangliosides in the chloroform phase was too low for accurate estimation, but in no test did it exceed 5 %.

After this method had been used for some time, SPERRY (1955) reported that calcium chloride could be used for freeing serum lipids from contaminants of low molecular weight. With the concentration used by him (20 mg $CaCl_2$/100 ml water), more gangliosides remained in the chloroform phase than with 100 mg NaCl/100 ml water as used by myself. When the calcium chloride concentration was increased, the ganglioside was content in the chloroform phase increased also.

Total lipids can be determined by evaporating the chloroform phase and weighing the residue. In the washing of the lipid phase, some of the saline will remain, and the higher the salt concentration, the higher the error that may be expected. SPERRY's method seems to be superior if the total lipids are to be determined gravimetrically. If, however, cerebrosides and gangliosides are to be estimated, the partition method described is suitable.

Hydrolysis of the Cerebrosides

Before hydrolysis can be performed, the solvent must be removed. On account of the amount of water in the chloroform phase, there is a great risk of bumping if the

solvent is removed by heating on a steam bath only. This was avoided by letting a stream of nitrogen remove the solvent. For the hydrolysis of cerebrosides, both hydrochloric acid and sulphuric acid have been used. It is more convenient to use the latter if a furfural method is used. But BRÜCKNER (1941), using 3 N sulphuric acid for the hydrolysis of pure cerebrosides, had a yield of only 94%. He suggested that the sugar released was destroyed by the sulphuric acid. A certain transformation of the hexose to furfural derivatives may occur during hydrolysis, but it will be of little importance, as the heating with the orcinol reagent will be long enough to give optimal furfural formation in the method described.

To avoid possible losses of furfural derivatives during hydrolysis, tubes with ground stoppers were used.

The optimal conditions for hydrolysis were determined on a phosphorus-free cerebroside preparation from human brain made according to KLENK and LEUPOLD (1944). The concentration of sulphuric acid and the heating time were varied. The same values were found with 3 h heating and 2 N acid and with 2 h heating and 3 N acid. The amount of hexose in the cerebroside preparation tested was found to be 21·8%, which agrees well with the theoretical figure (21·4–22·2%). This shows that no demonstrable destruction occurs during hydrolysis.

Removal of Fatty Acids

During the hydrolysis the fatty acids are split off, and have to be removed as they would interfere with the colorimetric reading. BRUCKNER (1941) removed them by extraction with chloroform, as he believed that the use of filter papers would introduce an error. Parallel runs with chloroform extraction and filtering gave identical results, but the standard error in the first method was higher. The filtration method was also more rapid to carry out, so it was preferred. The quality of the filter papers must be good, and the need for careful washing must not be forgotten.

COLOUR REACTION

Two methods have been tried for the determination of the sugar content in the hydrolysed samples, the anthrone and the orcinol methods. The anthrone method is very convenient and satisfactory when only one sugar in the sample has to be analysed, but the absorption coefficient is much lower for galactose than glucose, and the absorbency for a mixture of monosaccharides is lower than the sum of the absorbencies of the single sugars. The orcinol method has previously been used in the analysis of mixtures of sugars (SÖRENSEN and HAUGAARD, 1933; VASSEUR, 1948). The orcinol method of VASSEUR (1948) was therefore adopted, but it was soon evident that the conditions of colour formation were not optimal. Several factors influencing the colour formation were therefore tested. They were studied with a solution of 50 μg galactose with a heating time of 20 min at 80°C. The absorbency was measured in a Beckman-B spectrophotometer at 505 mμ against an orcinol–sulphuric acid blank.

Sulphuric Acid Concentration

The concentration of sulphuric acid was varied to produce a final concentration of between 15–30 N. As can be seen from Fig. 1, the optimal absorbency was obtained when the final concentration was about 25 N. This concentration can be obtained by mixing 4 ml concentrated acid with 2 ml water. It seemed best to dissolve the

orcinol in concentrated sulphuric acid, if the reagent was stable. This would diminish the time for pipetting in comparison with VASSEUR (1948) and BRÜCKNER (1955), whose orcinol reagent has a comparatively low acid concentration, so that they have to add concentrated sulphuric acid separately to each sample. The final acidity would be much higher than in the method of VASSEUR (1948), which was only 19 N. BRÜCKNER (1955) has also found that the final sulphuric acid concentration must be about 25 N to give maximal colour. The same optimal acidity was also found in the anthrone method.

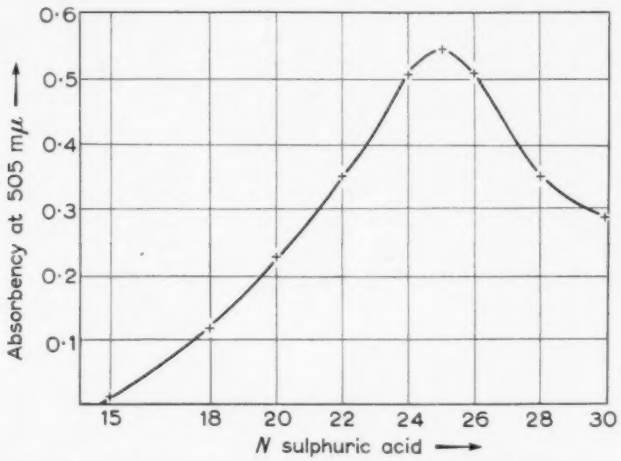


FIG. 1.—The absorbency–sulphuric acid concentration relationship over the range 15–30 N. Wavelength 505 mμ.

The reagent was stable for at least fourteen days when stored in a brown bottle in the refrigerator. In one month little or no decrease of the molar absorbency occurred.

Orcinol Concentration

The concentration of orcinol was varied in the reagent between 0·1 and 1·0%. The reading of the samples was done against a reagent blank with the same orcinol concentration as in the samples. Maximal absorbency was obtained with 0·3% orcinol, and then it decreased slowly down to 0·8%, where the drop was more rapid. Since the difference in absorbency between 0·2% and 0·3% orcinol was not more than 1%, the lower amount was used.

The Stability of the Colour Formed

After heating, the rack with the tubes was placed in an ice bath. The samples could not be read immediately, because the cuvettes became coated with moisture. They were therefore left at room temperature for at least 10 min before reading. To test the stability of the colour, they were placed at room temperature in a bright light. No change of absorbency occurred in two hours.

Influence of the Sugar Concentration

The validity of BEER's law has been rather limited for many of the methods used both with orcinol and anthrone reagents (see, e.g., SÖRENSEN and HAUGAARD, 1933;

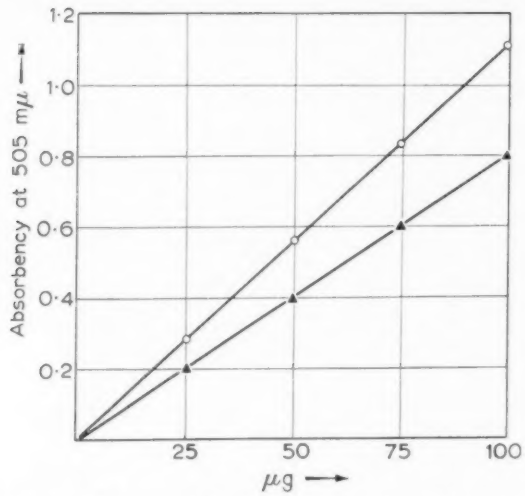


FIG. 2.—The absorbency–carbohydrate concentration relationship over the range 25–100 μg. —○—○, galactose; —▲—▲—, glucose. Each of the plotted values represents the mean of ten estimations.

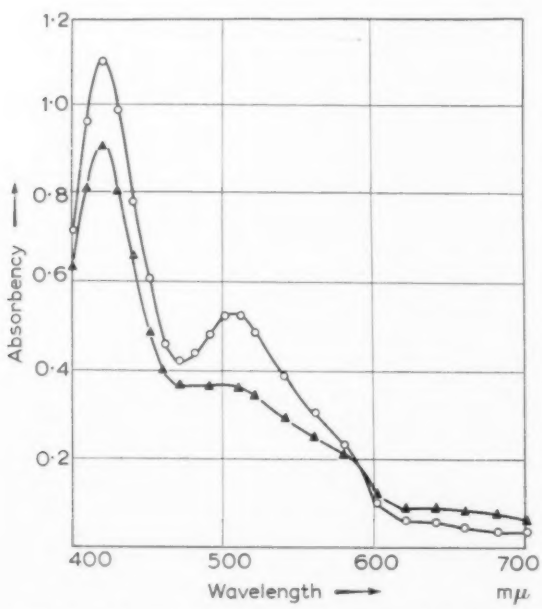


FIG. 3.—Absorption spectra of galactose and glucose (50 μg). —○—○— galactose; —▲—▲— glucose.

VOL 1 1956/

ROE, 1955). The method was tested with amounts from 10–100 μg galactose, mannose, and glucose. As will be seen from Fig. 2, the law was valid up to this concentration. Higher amounts of unknown samples have not been used, and if the absorbency was above 1·0, the sample was first diluted.

Absorption Spectra of Individual Sugars

The orcinol method has been used by several authors to estimate individual monosaccharides in a solution containing only one or two. This is possible by using different heating times and comparing the absorption curves (HAUGAARD and SÖRENSEN, 1933; VASSEUR, 1948; BRÜCKNER, 1955). With short heating times, various colours, ranging from yellow to bluish-red, are obtained with different sugars, and therefore also different absorption maxima. For my purpose it was,

TABLE 1.—THE ABSORBENCE OF GALACTOSE AND GLUCOSE AT 505 mμ BY THE METHOD OF EDGAR (1955)

Amount of sugar in μg	Galactose	Glucose
0	0·043	0·043
50	0·551	0·211
100	0·983	0·527

however, more useful to obtain as identical an absorbency as possible for the monosaccharides which may be found in a lipid extract from nervous tissue. Chromatographic analyses indicated only two, galactose and glucose. Both have, after 20 min heating, a sharp maximum at 420 mμ; but galactose has a second and smoother one at 505 mμ, while glucose only has one with a plateau with maximum about 500 mμ (Fig. 3). The ratio of the absorbency indices was, however, for galactose/glucose 1·4 at 505 mμ, which will lead to lower total sugar values if they are estimated together in a mixture and the sugars are calculated as galactose. In the chloroform extract obtained after partition, only traces of glucose were found, so in this investigation the total values for hexose should be valid.

EDGAR (1955) has suggested that glucose gives no colour with the orcinol method used by him (BRÜCKNER, 1941), but the method performed exactly as described gave an absorption ratio galactose : glucose of about 2 : 1, Table 1. If the orcinol method is used on an extract containing both glucose and galactose, the method must be combined with quantitative paper chromatography or a similar method to obtain the ratio between the two sugars. This method has also been used in an investigation of the polysaccharides of the nervous system, and has proved to be satisfactory (SVENNERHOLM and SVENNERHOLM, to be published). In this investigation it was also necessary to investigate the absorbency of other carbohydrates, the figures for which are given in Table 2.

The Use of a Sample Blank

The hydrolysed and filtered samples often had a slight yellow colour, the cause of which is unknown. It was much more prominent if the partition of the lipid

extract was not carried out. In this case it was attributable to water-soluble chromogens and sialic acid (neuraminic acid) in the gangliosides. EDGAR (1955) did not observe this with sialic acid, but it was clearly shown both with pure gangliosides and with sialic acid. FOLCH *et al.* (1951) based their determination of strandin (ganglioside) on the fact that a brown colour develops when strandin is heated with

TABLE 2.—ABSORBENCY VALUES OF CARBOHYDRATES WITH ORCINOL-H_2SO_4 REAGENT

Sugar	Absorbency value
Galactose	0·536
Glucose	0·371
Mannose	0·439
Fructose	0·316
Ribose	0·413
Glucuronic acid	0·389
Fucose	0·311

Carbohydrate solutions (50 μg/2 ml) were heated with the reagent 20 min at 80°C. Absorbency values were read at 505 mμ in a Beckman-B spectrophotometer against an orcinol–sulphuricacid blank.

hydrochloric acid. It was thought at first that this coloration was due to the decomposition of the sugar. Experiments with added galactose and cerebrosides gave 100% recovery, however.

As a matter of routine, a sample blank was therefore used both for the standards and the samples and subtracted from the test samples. The absorbency of the sample blank seldom exceeded 2% of that of the test after partition, but if lipid extracts were hydrolysed directly, the absorbency was about 10%, if the ganglioside content was high.

Reproducibility

The method described was used in an investigation of the glycolipid distribution in human brains (SVENNERHOLM, 1955b). The cerebrosides were estimated both by this method and by subtraction of the sugar in the gangliosides (calculated from estimations of hexosamine and sialic acid) from the total lipid-sugar. The statistical analysis of the correlation between the two methods is not finished, but in senile brains, for example, the same mean value of the amount of cerebroside in cerebral cortex was found by the two methods.

Determinations in duplicate on forty different lipid hydrolysates gave a standard error of 1·5%. The standard error of the whole method calculated on twenty duplicates of cerebral cortex was $\pm$4·2%. The amount of cerebrosides in the extracted material was about 0·8 mg. When the method was applied to foetal brains, the error was increased to about $\pm$6%, owing to the low content of cerebrosides.

DISCUSSION

As EDGAR (1955) has pointed out, accurate methods are needed for analysing small amounts of cerebrosides in nervous tissues. Gangliosides, which also contain

sugars, seem to be located mainly in the neurons (KLENK and LANGERBEINS, 1941; BRANTE and SVENNERHOLM, 1949, 1951), while cerebrosides are chiefly myelin sheath substances (BRANTE, 1949).

It has generally been assumed that the total quantity of lipid sugar is cerebroside sugar, so that the total amount of cerebrosides has been obtained by multiplying the sugar value by 4·6. In "white matter" the error introduced is small, since the ratio of cerebrosides to gangliosides is greater than 20 to 1. But in tissues containing nerve cells, e.g., cerebral cortex, where myelinated structures are scanty, the ratio of cerebrosides to gangliosides is about 1 (SVENNERHOLM, 1955b), and thus the value for cerebrosides is 100% too high. In analysis of foetal tissues, the error is even greater. In pathological material a decrease in the amount of cerebrosides may be masked by a corresponding increase in the gangliosides (EDGAR, 1955). Where a great increase in the gangliosides has occurred, a rough correction for the amount of gangliosides has as a rule been made. But smaller differences in the glycolipid ratio have not been found, since no suitable method has been available.

There are two possible procedures for the quantitative estimation of cerebrosides. In the first, the cerebroside sugar is determined by subtracting ganglioside sugar from the total lipid sugar. The amount of ganglioside is determined by estimating the amount of hexosamine or sialic acid, and from these values the amount of sugar in the gangliosides can be derived, if the ratio of hexosamine to sugar or of sialic acid to sugar in gangliosides is known. In the second procedure the glycolipids are separated and the amount of sugar is determined in the cerebroside fraction.

The first procedure can be used only under the following conditions:

1. The lipid extract must contain cerebrosides and gangliosides as the only sugar-containing compounds.
2. The carbohydrate estimation ought to be specific for hexose and give about the same results for galactose and glucose.
3. A convenient and accurate method for the determination of gangliosides must be available.
4. The composition of the gangliosides must be established.

1. FOLCH *et al.* (1951b) have found that in most extraction methods low-molecular substances contaminate a lipid extract, and it is impossible to remove them completely without some loss of gangliosides.

2. The estimation of the carbohydrate content in the lipid hydrolysate may be performed by methods based on the reducing power in strong alkaline solution or the formation of furfural derivatives in strong acid solution. Reducing methods are rather unspecific, and the reducing value for gangliosides will depend on the amount of hexoses, hexosamine, and sialic acid. Nor is the latter type of method altogether satisfactory, as the molar absorbency indices are different for the common hexoses. But in a lipid extract the amount of galactose is considerably higher than that of glucose, so that the error introduced by the different molar absorbencies will be small.

3. Gangliosides may be determined as sialic acid (neuraminic acid) with Bial's test by the method of KLENK and LANGERBEINS (1941), or as hexosamine by a modified Morgan-Elson method (1933) (BRANTE and SVENNERHOLM, 1951). Bial's test for sialic acid is influenced by a lot of factors, and in the presence of a large amount of hexose and/or fatty acids may give quite erroneous results (SVENNERHOLM, 1955a). The hexosamine method also may give errors of about 40% (BOAS, 1953) when used on nervous tissue. The ganglioside values may therefore be very uncertain when the methods are used on total lipid extracts.

4. The composition of the polysaccharide part of the gangliosides changes during development (SVENNERHOLM, 1955a), which also reduces the possibility of calculating the amount of gangliosides.

The second procedure depends entirely on the possibility of separating the gangliosides and cerebrosides. If a partition method is used, low-molecular contaminants will accompany the gangliosides. In the method used in the present investigation the separation was satisfactory for amounts of tissue between 10 and 60 mg (the smaller amounts were used when high glycolipid values were expected). When the total amount of cerebrosides was high, the partition was delayed and a smaller part of the cerebrosides went into the aqueous phase. In pathological material, where the amount of gangliosides is greatly increased, part of the gangliosides may remain in the lipid phase.

Independently of each other, EDGAR (1955) and SVENNERHOLM (1955b) have investigated the amount of cerebrosides in brains before visible myelination. EDGAR used the first procedure, with brains from rabbits 0 and 9 days old. He found that all the hexose determined derived from the gangliosides and, although the number of analyses was small, the general tendency was for the hexose content to be considerably lower than might be expected from the determined quantities of neuraminic acid and hexosamine. This illustrates the difficulties in obtaining reliable results with this type of method.

In my own investigation, brains from human foetuses between 4 and 6 months of age were used. By both the partition dialysis method of FOLCH *et al.* (1951b) and the method described above, cerebrosides were clearly demonstrated (SVENNERHOLM, 1955b).

The divergence in the results of the two investigations may depend on several factors, such as the different materials used and the splitting of the gangliosides in the human material during storage, as well as the reliability of the methods used.

The question of whether the cerebrosides in the brain are only myelin lipids or whether they also occur in the nerve cells is, however, of such great theoretical and physiological importance that further investigations are necessary. This problem cannot be solved before attempts have been made to isolate the cerebrosides from nervous tissue free from myelin, e.g., foetal brain. Investigations with this object are proceeding, even though SCHUWIRTH (1940) was not able to isolate cerebrosides from foetal brains.

SUMMARY

1. A method for the quantitative estimation of cerebrosides in nervous tissue based on the partition method of FOLCH *et al.* (1951b) is described.

2. The hexose content of the cerebrosides is determined by an orcinol-sulphuric acid method. The optimal conditions for this method are investigated.

3. With the older methods the figures obtained for cerebrosides include gangliosides. The possibility of calculating the cerebroside content by subtracting ganglioside sugar from total lipid sugar is critically discussed.

Acknowledgements—This work has been supported by a grant from the Medical Faculty, University of Gothenburg, and by "Göteborgs Läkaresällskap." The skilful assistance of Mrs. BIRGIT JOHANSSON and Mrs. AINA RAAL is gratefully acknowledged.

REFERENCES

BOAS N. F. (1953) *J. Biol. Chem.* **204,** 553.
BRAND F. C. and SPERRY W. M. (1941) *J. Biol. Chem.* **141,** 545.
BRANTE G. (1949) *Acta Physiol. Scand.* **18,** supp. 63.

BRANTE G. (1951) *Fette und Seifen* **53,** 457.
BRANTE G. and SVENNERHOLM L. See BRANTE (1949), p. 172, and BRANTE (1951).
BLACKWOOD W. and CUMINGS J. N. (1954) *J. Neurol. Neurosurg. Psychiat.* **17,** 33.
BRÜCKNER J. (1941) *Z. physiol. Chem.* **268,** 163.
BRÜCKNER J. (1955) *Biochem. J.* **60,** 200.
CHATAGNON C. and CHATAGNON P. (1953) *Bull. soc. chim. biol.* **35,** 1319.
CHATAGNON C. and CHATAGNON P. (1954) *Bull. soc. chim. biol.* **36,** 373.
CUMINGS J. N. (1953) *Brain* **76,** 551.
DAUN H. (1952) Thesis, Köln.
DISCHE Z. (1930) *Mikrochimie* **8,** 4.
EDGAR G. W. F. (1955) Thesis, Amsterdam. To be published in *Acta Anatomica* **27,** 240.
EDMAN P. V. (1942) *J. Biol. Chem.* **143,** 219.
FOLCH J., ARSOVE S., and MEATH J. A. (1951a) *J. Biol. Chem.* **191,** 819.
FOLCH J., ASCOLI T., LEES M., MEATH J. A., and LEBARON F. N. (1951b) *J. Biol. Chem.* **191,** 833.
HACK M. H. (1952) *Biochem. J.* **54,** 602.
JOHNSON A. C., MCNABB A. R., and ROSSITER R. J. (1950) *Arch. Neurol. Psychiat.* **64,** 105.
KLENK E. (1941) *Z. Physiol. Chem.* **268,** 50.
KLENK E. (1942) *Z. Physiol. Chem.* **273,** 76.
KLENK E. and LANGERBEINS H. (1941) *Z. Physiol. Chem.* **270,** 185.
KLENK E. and LEUPOLD F. (1944) *Z. Physiol. Chem.* **281,** 208.
MILLER B. F. and VAN SLYKE D. D. (1936) *J. Biol. Chem.* **114,** 583.
NOLL A. (1899) *Z. physiol. Chem.* **27,** 370.
ROE J. H. (1955) *J. Biol. Chem.* **212,** 335.
SCHUWIRTH K. (1940) *Z. Physiol. Chem.* **263,** 25.
SPERRY W. M. (1954) *J. Biol. Chem.* **209,** 337.
SPERRY W. M. and BRAND F. C. (1955) *J. Biol. Chem.* **213,** 69.
SVENNERHOLM L. (1955a) *Congr. of Neurolipidosis, Antwerpen.*
SVENNERHOLM L. (1955b) 3. *Int. Congr. Biochem.*, p. 81.
SVENNERHOLM L. (1955c) *Acta Chem. Scand.*, **9,** 1033.
SVENNERHOLM E. and SVENNERHOLM L. To be published.
SÖRENSEN M. and HAUGAARD G. (1933) *Biochem. Z.* **260,** 247.
VASSEUR E. (1948) *Acta Chem. Scand.* **2,** 693.

VOL. 1 56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 54-67, Pergamon Press Ltd., London

THE QUANTITATIVE HISTOCHEMISTRY OF THE CEREBRAL CORTEX—I

ARCHITECTONIC DISTRIBUTION OF TEN CHEMICAL CONSTITUENTS IN THE MOTOR AND VISUAL CORTICES*

ELI ROBINS, DAVID E. SMITH, and KATHRYN M. EYDT
Department of Psychiatry and Neurology and the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri

(*Received* 11 *January* 1956)

VOL
1
1956/

THE development of quantitative methods for the measurement of chemical and enzymatic tissue constituents in samples of tissue weighing less than 10 μg has made possible the study of the amount and localization of these constituents at rather precise locations in the brain (ROBINS and SMITH, 1953; LOWRY, ROBERTS, LEINER, WU, FARR, and ALBERS, 1954; POPE, 1952). By utilization of these methods, it is possible to study the chemical and enzymatic architectonics of the cerebral cortex.

In this report, the results of a study of the distribution of dry weight per unit volume, protein, total lipide, cephalins, lecithins, sphingomyelins, total sphingolipides, non-phosphorus-containing sphingolipides, total phospholipides, cholesterol, and number of cells per unit volume in each histologically defined layer and sublayer of the motor cortex and visual cortex of the monkey are presented. In the accompanying papers, studies of some of the enzymatic constituents of these layers (ROBINS, SMITH, EYDT, and MCCAMAN, 1956) and of analyses at 50 μ intervals compared with those by architectonic layers throughout the depth of the two cortices (ROBINS, SMITH, and EYDT, 1956) are reported.

MATERIAL AND METHODS

Adult monkeys (*Macaca mulatta*), weighing from 3·5 to 4·7 kg, were used in this study. The motor and visual cortices were defined according to the locations and criteria recently described in the elegant study of VON BONIN and BAILEY (1947). The (precentral) motor cortex in this study is area *FA* of VON BONIN and BAILEY, and is the electrically excitable motor cortex containing Betz cells. Area *FA* is an example of agranular cortex. The visual (striate) cortex in this study is synonymous with area *OC* of VON BONIN and BAILEY and is an example of koniocortex. The criteria for defining the layers in stained sections of each cortex were those described by VON BONIN and BAILEY. The layers of the motor cortex are symbolized in this study as 1, 2, 3*o* (outer portion of layer 3), 3*i* (inner portion of layer 3), 4, 5-Betz (outer portion of layer 5 which contains Betz cells), 5*o* (outer portion of layer 5 lying between that containing Betz cells and the inner portion of layer 5), 5*i*, 6, and *W* (white matter from 25 to 150 μ subjacent to the cortex).† The layers of the visual cortex

*Aided by grants from the National Foundation for Infantile Paralysis and the National Institute of Mental Health, United States Public Health Service. A preliminary report of these findings has appeared (ROBINS and SMITH, 1954).

†Layer 3*o* corresponds to layer *iiia* + *b* of VON BONIN and BAILEY; layer 3*i* to layer *iiic*; layers 5-*Betz* and 5*o* to layer *va* + *b*; and layer 5*i* to layer *vc*. Our use of the terms outer and inner follows that of VON BONIN and BAILEY. The direction perpendicular to the layers and to the pial surface is denoted as outer and inner. Outer is defined as nearer the pial surface, and inner as nearer the white matter of the hemisphere.

are symbolized in this study as 1, 2, 3*o*, 3*i*, 4*o*, 4*i*, 5, 6*o*, 6*i*, and *W**. Layers 4*o* and 5 of the striate cortex correspond to the outer line of Baillarger (line of Gennari) and the inner line of Baillarger, respectively (VON BONIN and BAILEY, 1947; O'LEARY, 1941; VON BONIN, 1942).

The animals were sacrificed by decapitation without anaesthesia. The skull was opened rapidly and the two cerebral hemispheres were separated longitudinally; the brain stem and part of the basal ganglia were removed from each hemisphere, and the hemispheres were plunged into liquid nitrogen. Only seven to ten minutes elapsed from the death of the animal until the cerebral hemispheres were immersed in liquid nitrogen. The tissue was then allowed to warm to −20°C in a cold room, and the motor and visual cortices were undercut approximately 6 to 8 mm below the pial surface. Using a cork borer or similar instrument with a sharp cutting edge, cylinders of cortex 3 mm in diameter from the lateral surfaces of the hemispheres were punched out by exerting gentle direct pressure (no turning motion) on the borer. The long axis of these cylinders was perpendicular to the pial surface. An attempt was made to take the cylinders at the crowns of the gyri, *i.e.* as far away from a sulcus as possible, otherwise the deeper layers of the cortex would not be absolutely perpendicular to the long axis of the cylinder (this point was checked by staining the lower border of the cortex—see below). This requirement forced us to discard, after sectioning, approximately one-fifth of the cylinders. The cylinders were mounted and cut into sections 25 μ thick, as described by LOWRY (1953), without the temperature ever being allowed to rise above −15°C. The cut sections were placed serially in special holders (LOWRY, 1953). The bottom of the cortex was found by inspection of selected sections stained with 1% thionine or cresyl violet. It was possible to locate the junction of layer 6 and white matter by finding three sections, each 50 to 100 μ apart, of which the outer one had a moderate number of neurons, the middle one had only a very few neurons, and the inner one was entirely white matter†. The bottom of the cortex was then taken as the next section below the middle one. It was at this stage that many cylinders had to be discarded because tangential orientation of the cylinders had resulted in these last sections being approximately half white matter and half layer 6. If a section at the junction of layer 6 and white matter showed a rim of neurons less than one-tenth the diameter of the whole section, this rim was discarded during dissection and the cylinder was used for the chemical studies. This occurred in two of the nine cylinders that were used for the analysis.

Once the sections were cut, they were dehydrated under vacuum (0·001 mm) overnight at a temperature not exceeding −35°C (LOWRY, 1953). After dehydration, the sections could be stored for months at −25°C without deterioration.

The extremely rapid freezing at −196°C, careful sectioning without compression, and dehydration at −35°C gave assurance that these tissues had retained their original volume (LOWRY, 1953). The true total depth of the cortex in μ was therefore ascertained by simply multiplying by 25‡ the number of sections from the pial surface to the inner edge of layer 6.

The position of each frozen-dried section in the architectonic layers of the cortex was determined by studying stained sections taken vertically from the brain adjacent to the site of the removal of the cylinder. Since tissue is known to shrink on fixation, embedding, and staining, it was necessary to prove that the shrinkage in each layer was proportional to that in the other layers and the whole specimen. After this had been satisfactorily demonstrated, as is described in the next paragraphs, the relative thickness of each layer compared to the total thickness of the cortex in the vertically stained sections was determined. From these ratios it was possible to divide the dried sections into groups corresponding to the layers of the cortex.

The block of tissue surrounding the punched-out cylinder was fixed in 4% formaldehyde for one to seven days, paraffin-embedded,§ and sectioned vertically at 20 μ. These vertical sections were then stained with thionine,¶ and their thicknesses measured with an eyepiece filar micrometer which

*Layer 4*o* corresponds to layer *iva* of VON BONIN and BAILEY; layer 4*i* to layer *ivb*; layer 6*o* to layer *via*; and layer 6*i* to layer *vib*.

†From five to ten other sections, reaching as high as layer 4, were stained in order to be certain that no aberrant patches of white matter were present at these more superficial levels.

‡In unpublished studies with Mr. IRVING J. WEIGENSBERG, we have shown that under these conditions the thickness of the microtome sections are within 5% (usually below) of 25 μ. The linear expansion of brain on freezing (LOWRY, 1953) is less than 2%, and this factor has been neglected in the calculations.

§Two blocks of visual cortex were fixed in 4% formaldehyde, cut vertically as frozen sections without embedding, and then stained (see footnote ¶.)

¶The sections were placed in 1% aqueous thionine for 30 min at 50°C, differentiated with 5% aniline in 95% ethyl alcohol, dehydrated through absolute alcohol into xylene, and mounted in balsam.

had been calibrated against a stage micrometer. From these measurements the fraction each layer comprised of the total thickness of the cortex was calculated. A comparison of the total depth of the cylinder with that of the stained vertical section gives a measure of the amount of shrinkage caused by the fixation, embedding, and staining. The data in Table 1 indicate that there was an enormous shrinkage (36%) on fixation and staining of the tissues. This was increased by a quarter (to 45%) if the tissue was also embedded in paraffin prior to sectioning. If this linear shrinkage is identical in amount in all three planes, these data would indicate a 76% and 83% shrinkage in volume, respectively. Although these values are very high, similar data have been obtained for the kidney on formalin fixation and paraffin-embedding (STOWELL, 1941).

TABLE 1.—SHRINKAGE OF CORTICAL TISSUE RESULTING FROM FIXATION, EMBEDDING, AND STAINING

Monkey No.	Cortical area	Depth of cortex (μ)			Shrinkage (%)	
		Frozen-dried*	Fixed, embedded, stained†	Fixed, stained‡		
1	Motor	3950	2170		45	
2a	Motor	3950	2100		47	
2b	Motor	3650	1870		49	
3	Motor	3600	2070		43	
	Mean shrinkage				46	
5	Visual	2050	1140		44	
6a	Visual	2050	1120		45	
	Mean shrinkage					45
6b	Visual	2100		1310	38	
6c	Visual	2100		1390	34	
	Mean shrinkage					36

*This depth was measured on the unfixed, unembedded, and unstained tissue as described in the text, by the number of 25-μ microtome sections from the pial surface to the junction of layer 6 and white matter.

†This depth was measured with an eyepiece filar micrometer on vertical sections treated as described in the text and footnote ¶ on page 55.

‡This depth was measured with an eyepiece filar micrometer on vertical sections treated as described in the text and footnote § on page 55.

It was possible to show that this shrinkage affected the layers of the cortex approximately equally. In the unstained frozen-dried sections from the cylinders of motor cortex it was possible to identify the junction of layers 1 and 2, and to identify layer 5-*Betz* by the presence of the Betz cells, which are visible in the unstained material. When the fraction of the total thickness of the stained vertical section occupied by layer 1 was multiplied by the depth of the cylinder, the calculated true thickness of layer 1 was found to coincide with the measured depth of the junction of layers 1 and 2 in the cylinder in all instances. When the same calculation was made for layer 5-*Betz*, a difference of 50 to 100 μ from the measured depth below the pia was usually found and no difference greater than 150 μ was found—a maximal error of less than 7% (mean depth of layer 5-*Betz* beneath pia = 2200 μ). It is, therefore, probable that the shrinkage of the stained vertical sections affected all layers of the cortex approximately equally. Since the shrinkage in all layers seemed to be approximately equal, an estimate of the true absolute thickness of each layer was calculated by multiplying the fraction of the total thickness each layer comprised of the vertically cut sections by the true total depth as ascertained on the frozen cylinder of cortical tissue.

The dissection of the frozen-dried tissue was done at room temperature under a dissecting microscope. Since each section was considered to be homogeneous, samples were taken at random from within a given section. The bits of tissue analysed varied in weight from 0·1 μg to 15 μg, depending on the constituent to be measured. For each layer, the middle 100 μ (four 25 μ sections) of that layer were used as a representative sample. In the narrower layers of the visual cortex, the middle 50 μ, in most instances, was used as a representative sample. For layer 1, the outer 100 to 150 μ (the pia was dissected and discarded) were used as a representative sample. For white matter, tissue 25 to 150 μ below the inner edge of layer 6 was used.

Methods for handling the small volumes and weights have been described (LOWRY, 1953; LOWRY, ROBERTS, LEINER, WU, and FARR, 1954).

Chemical methods. Methods for determining dry weight (LOWRY, 1953), volume (LOWRY, 1953), lipide fractions (ROBINS, LOWRY, EYDT, and MCCAMAN, 1956), protein (LOWRY, ROSEBROUGH, FARR, and RANDALL, 1951), and cholesterol (ALBERS and LOWRY, 1955), have been given. Deoxyribonucleic acid (DNA) was determined by a fluorimetric method, based on the method suggested by PESEZ (1950).* This method gives no reaction with ribonucleic acid and does not give falsely high values for DNA in brain, as has been found for some other chemical methods (LOGAN, MANNELL, and ROSSITER, 1952).

VOL. 1 56/57

RESULTS

Thickness of the Layers of the Motor and Visual Cortices (*Tables* 2 *and* 3)

These data on the thickness of the individual layers agree well with those of VON BONIN and BAILEY (1947). The mean depth of the entire motor cortex is almost twice that of the visual cortex, 3900 μ vs. 2100 μ. There was surprisingly little variation in the thickness of the individual layers or in the total depth of the cortex in the same animal or in different animals.

TABLE 2.—THICKNESS OF LAYERS OF THE MOTOR CORTEX

Cortical layer	Depth below pia of inner edge of each layer (μ)						Thickness of each layer (μ)	Percentage of total thickness of the cortex		
	Monkey 1	Monkey 2a	Monkey 2b	Monkey 3	Monkey 4	Mean	Mean	Present study		VON BONIN and BAILEY (1947)
1	250	250	200	250	200	250	250	6	6	5
2	550	550	450	500	450	500	250	6	6	6
3*o*	1050	1150	1050	1150	1150	1100	600	16	29	32
3*i*	1850	1850	1300	1450	1700	1600	500	13		
4	2000	2000	1450	1600	1850	1800	200	5*	5	9
5-*Betz*	2300	2250	1850	2000	2550	2200	400	10		
5*o*	2850	2850	2450	2450	3050	2750	550	14	30	26
5*i*	3050	3100	2700	2700	3500	3000	250	6		
6	3950	3950	3650	3600	4200	3900	900	23	23	22

* KISSANE, J. M., and ROBINS, E., unpublished.

TABLE 3.—THICKNESS OF LAYERS OF THE VISUAL CORTEX

Cortical layer	Depth below pia of inner edge of each layer (μ)					Thickness of each layer (μ)	Percentage of total thickness	
	Monkey 5	Monkey 6a	Monkey 6b	Monkey 6c	Mean	Mean	Present study	VON BONIN and BAILEY (1947)
1	150	150	200	200	150	150	7	4
2	350	400	400	400	400	250	12	7
3*o*	550	650	650	750	650	250	12	33
3*i*	850	900	900	1000	900	250	12	
4*o*	1150	1250	1200	1300	1200	300	14	25
4*i*	1450	1500	1500	1550	1500	300	14	
5	1750	1700	1700	1750	1750	250	12	12
6*o*	1900	1950	1900	1950	1900	150	7	19
6*i*	2050	2050	2100	2100	2100	200	9	

Protein Content and Dry Weight per Litre (*Tables* 4 *and* 5)

The *dry weight per litre* was relatively constant in both motor and visual cortices from layers 1 through 5-*Betz* and 5, respectively. There was a slight increase in layers 5*i* and 6*o*, respectively, and a marked increase in layer 6 and white matter.

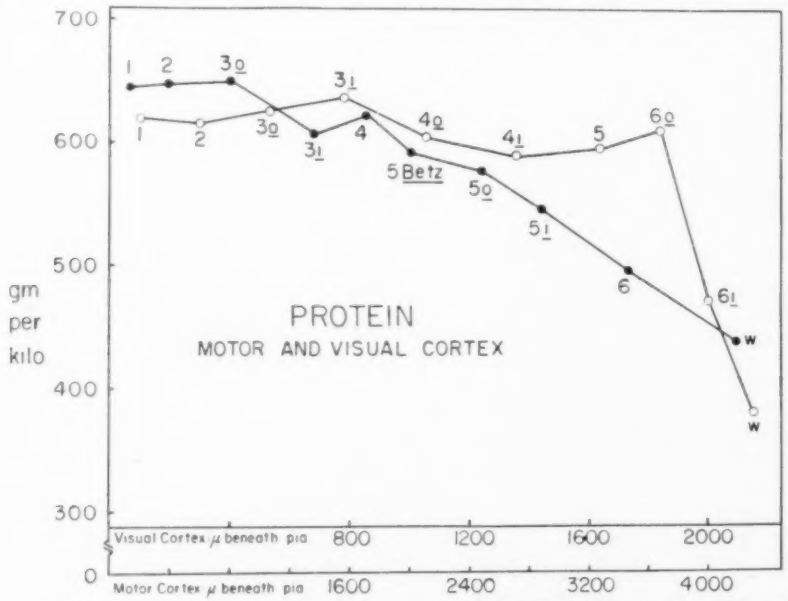


FIG. 1.—Protein content in the layers of the motor (●) and visual (○) cortices, showing characteristic pattern described in the text.

These values are similar to those reported for the white matter and the various layers of cerebellar cortex in the monkey (ROBINS, EYDT, and SMITH, 1956) and Ammon's horn in the rabbit (LOWRY, ROBERTS, LEINER, WU, FARR, and ALBERS, 1954).

TABLE 4.—DRY WEIGHT, PROTEIN, TOTAL LIPIDE, DNA, AND NUMBER OF CELLS OF THE MOTOR CORTEX OF THE MONKEY

Protein values are averages of twelve to twenty-one measurements for each layer on three animals. Dry weight and DNA values are averages of three to ten measurements for each layer on one animal.

Cortical layer	Dry weight (1)	Protein (2)	Total lipide* (3)	DNA phosphorus (4)	DNA† (5)	Total cells‡ (6)
	gm per l	% dry weight	% dry weight	millimoles per kilo dry weight	% dry weight	Number per mm^3
1	229	64·5	32·6	6·8	0·28	90,000
S.e.m.§	6	1·0		0·6		
2	239	64·7	33·5	7·7	0·32	108,000
S.e.m.	4	1·2		0·3		
3*o*	192	64·9	35·9	9·0	0·37	100,000
S.e.m.	9	0·7		0·4		
3*i*	194	60·6	41·3	11·3	0·47	128,000
S.e.m.	10	0·8		0·3		
4	243	62·0	42·2	10·8	0·45	153,000
S.e.m.	6	1·4		0·2		
5-*Betz*	207	59·1		11·2	0·46	134,000
S.e.m.	7	1·1		0·3		
5*o*		57·6	43·5	9·9	0·41	
S.e.m.		1·3		0·3		
5*i*	253	54·4	45·7	10·6	0·44	156,000
S.e.m.	3	1·2		0·3		
6	295	49·3	49·1	8·8	0·36	149,000
S.e.m.	7	0·7		0·4		
W	378	43·7	55·4	5·7	0·24	128,000
S.e.m.	10	0·6		0·5		

*Calculated from the data in columns 7 and 8 in Table 6, using 385 as the molecular weight of cholesterol and assuming a molecular weight of 800 for cephalins, lecithins, and sphingolipides (BRANTE, 1949).

†Calculated from the data in column 4. The DNA standard (Worthington Biochemical Corp.) had a 7·5 per cent phosphorus content.

‡Calculated from the data of HELLER and ELLIOT (1954). Each nucleus contains $7{\cdot}1 \times 10^{-12}$ gm DNA.

§S.e.m. = Standard error of the mean.

The protein content of motor cortex (Table 4 and Fig. 1) was relatively constant in layers 1, 2, and 3*o*, but began to decrease in layer 3*i* and continued to do so into the white matter. The protein content of visual cortex (Table 5 and Fig. 2) showed a quite different pattern. It was almost constant from layers 1 through 6*o* and then

TABLE 5.—DRY WEIGHT, PROTEIN, TOTAL LIPIDE, DNA, AND NUMBER OF CELLS OF THE VISUAL CORTEX OF THE MONKEY

Protein values are averages of eight to fifteen measurements for each layer on three animals. Dry weight and DNA values are averages of three to nine measurements for each layer on one animal.

Cortical layer	Dry weight (1)	Protein (2)	Total lipide* (3)	DNA phosphorus (4)	DNA† (5)	Total cells† (6)
	gm per l	% dry weight	% dry weight	millimoles per kilo dry weight	% dry weight	Number per mm³
1	208	61·7	31·0	10·7	0·44	128,000
S.e.m.‡	9	1·4		0·7		
2	221	61·4	28·5	18·0	0·74	231,000
S.e.m.	9	0·8		0·3		
3*o*	194	62·4	31·9	17·6	0·73	200,000
S.e.m.	6	0·7		0·3		
3*i*	205	63·6	31·0	19·8	0·82	237,000
S.e.m.	7	0·6		0·5		
4*o*	191	60·2	34·9	18·0	0·74	200,000
S.e.m.	10	0·6		0·8		
4*i*	191	58·8	34·3	24·5	1·02	274,000
S.e.m.	4	0·6		1·8		
5	201	59·3	35·2	22·5	0·93	263,000
S.e.m.	6	0·6		0·8		
6*o*	253	60·7	40·6	15·9	0·66	235,000
S.e.m.	8	1·1		1·1		
6*i*	346	47·1	45·1	11·0	0·45	225,000
S.e.m.	14	2·0		0·8		
W	354	38·1	56·7	6·7	0·28	139,000
S.e.m.	10	1·0		0·3		

*Calculated from the data in columns 7 and 8 in Table 7, using a molecular weight of 385 for cholesterol and assuming a molecular weight of 800 for cephalins, lecithins, and sphingolipide (BRANTE, 1949).

†Calculated as described in the second and third footnotes to Table 4.

‡S.e.m. = Standard error of the mean.

abruptly decreased in 6*i* and white matter. These contrasting patterns in the two cortices will be seen—see below and Paper II (ROBINS, SMITH, EYDT, and MCCAMAN, 1956)—to obtain for many other constituents of the two cortices. It is especially noteworthy that in layers 4*o* and 5 of the visual cortex (lines of Baillarger), where there is apparently much more myelin as inferred from the grossly visible line of Gennari and the occurrence of osmic-acid staining material, there is no appreciable decrease in the proportion of protein. Despite this difference in the pattern of distribution in the two cortices, the absolute amounts of protein in the motor and visual cortices are similar at their maxima, 65% and 64%, and at their minima, 44% and 38%, respectively.

VOL 1 1956/

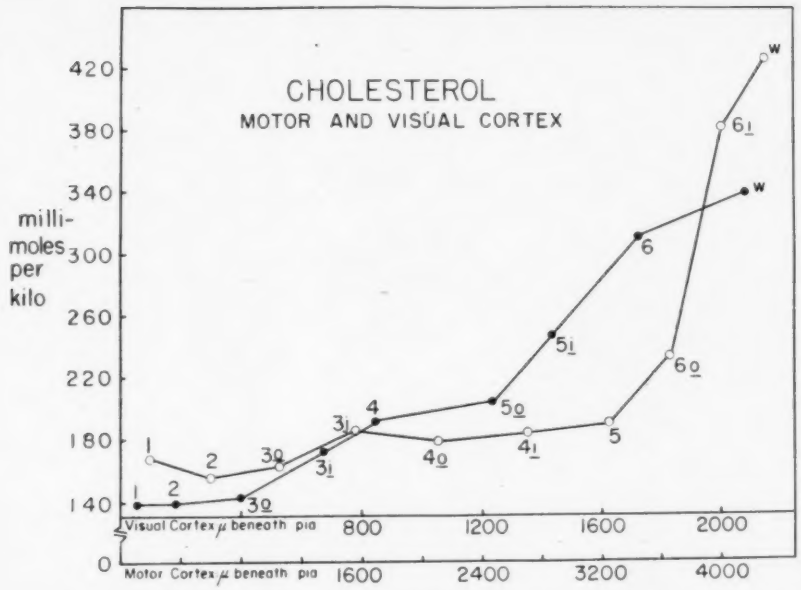


FIG. 2.—Cholesterol content in the layers of the motor (●) and visual (○) cortices, showing characteristic pattern described in the text.

Total Lipide and Lipide Fractions

The *total lipide* of motor cortex was relatively low in the outermost layers: it rose slightly but definitely in layer 3*i*, then continued to increase gradually for a few layers, and finally reached its peak in white matter. In the visual cortex, total lipide was low in the outermost layers, increased slightly at 4*o*, and then remained constant until it began to increase abruptly through the layers of 6 and into white matter. The absolute amounts of total lipide in the two cortices are similar, varying from a minimum of 29 to 32% of the dry weight to a maximum of 55 to 57%. In the visual cortex, layers 4*o* and 5 (lines of Baillarger) do not have significantly different total lipide from the intervening layer 4*i* or the subjacent layer 5. This finding, as well as that of the protein content (see above), suggests that visible myelination in these layers is not associated with the expected decrease in protein content and increase in total lipides found in white matter.

That there is something unusual about the increased concentration of visible myelin in these layers 4*o* and 5 of visual cortex is also indicated by the findings on *cholesterol* (Table 7). Cholesterol has been suggested as being a lipide characteristic of myelin (JOHNSON, MCNABB, and ROSSITER, 1948), which has been confirmed (BRANTE, 1949; ROBINS, EYDT, and SMITH, 1956). Even cholesterol, however, showed no appreciable increase in these two layers. On the other hand, cholesterol did show a distribution which was almost a mirror image of the distribution of protein (Tables 6 and 7 and Figs. 1 and 2) in both the motor and visual cortices, which is consistent with its being an index of the amount of myelin. It is probably the best index available to us, because cerebrosides, which would be a better index (FOLCH-PI, 1955), are not determined as such by our micromethods. Instead, we measure non-phosphorus-containing sphingolipides, which include chiefly cerebrosides and (in

TABLE 6.—LIPIDE FRACTIONS OF THE MOTOR CORTEX OF THE MONKEY

All values expressed as millimoles per kilo dry weight. Each value is the mean of four to nine determinations on one animal.

Cortical layer	Cephalins (1)	Lecithins (2)	Sphingomyelins (3)	Total sphingolipides (4)	Non-phosphorus-containing sphingolipides (4) − (3) (5)	Total phospholipides (1)+(2)+(3) (6)	Total phospho- + sphingolipides (1)+(2)+(4) (7)	Cholesterol (8)
1	143	126	50	72	22	319	341	139
S.e.m.*	5	10	2	7	6	6	11	7
2	147	125	45	82	37	317	354	138
S.e.m.	15	18	2	2	5	5	9	4
3*o*	153	122	40	105	65	315	380	143
S.e.m.	4	5	1	4	3	5	8	5
3*i*	168	104	41	162	121	313	434	171
S.e.m.	4	7	2	4	7	6	6	6
4	159	126	50	150	100	335	435	192
S.e.m.	5	7	3	12	13	8	13	5
5-*Betz*	155	107	39	168	129	301	430	
S.e.m.	10	10	2	15	18	4	18	
5*o*	159	116	47	170	123	322	445	204
S.e.m.	6	5	4	7	8	6	8	5
5*i*	161	113	50	179	129	324	453	247
S.e.m.	3	4	4	5	7	5	6	11
6	179	101	58	184	126	338	464	311
S.e.m.	8	3	1	17	15	9	20	16
W	210	87	91	236	145	388	533	329
S.e.m.	13	6	3	27	24	11	30	14

*S.e.m. = Standard error of the mean.

lesser amounts) gangliosides. The cerebrosides are confined almost exclusively to white matter (ROBINS, EYDT, and SMITH, 1956; FOLCH-PI, 1955), whereas the gangliosides probably occur only in neurons. Thus, our values for non-phosphorus-containing sphingolipides include a lipide (gangliosides) not characteristic of myelin.

The distribution of the *non-phosphorus-containing sphingolipides* shows that they are probably the most characteristic lipide group of white matter (*i.e.* highest ratio of concentration in white matter to that in grey) in both cortices (Tables 6 and 7) and in the cerebellum (ROBINS, EYDT, and SMITH, 1956). Also, as was previously found in the study of the cerebellum, these lipides are present in surprisingly low concentrations in the molecular layer of both cortices. In the visual cortex, this very low concentration apparently extends as deep as layer 3*i* (Table 7). In the motor cortex the increase in going from 3*o* to 3*i* probably indicates a relatively heavy deposit of myelin in 3*i* which is not present in this concentration at a more superficial level and which remains or increases in the deeper layers. This suggestion is supported by the same change which occurs in cholesterol in going from 3*o* to 3*i* and the inverse change which occurs in the protein content (Figs. 1 and 2). Thus, in motor cortex,

VOL 1 1956/

TABLE 7.—LIPIDE FRACTIONS OF THE VISUAL CORTEX OF THE MONKEY

All values expressed as millimoles per kilo dry weight. Each value is the mean of four to nine determinations on one animal, except for total sphingolipides in layer 1, for which only one determination was made.

Cortical layer	Cephalins (1)	Lecithins (2)	Sphingomyelins (3)	Total sphingolipides (4)	Non-phosphorus-containing sphingolipides (4)−(3) (5)	Total phospholipides (1)+(2)+(3) (6)	Total phospho- + sphingolipides (1)+(2)+(4) (7)	Cholesterol (8)
1	136	116	37	44	9	289	296	168
S.e.m.*	9	7	2			10		8
2	121	127	30	33	3	278	281	155
S.e.m.	9	5	3	3	3	13	16	6
3*o*	142	126	33	43	10	301	311	162
S.e.m.	3	9	3	5	6	9	5	3
3*i*	143	119	33	36	3	295	298	186
S.e.m.	20	12	3	7	6	11	13	6
4*o*	144	128	35	78	43	307	350	179
S.e.m.	7	2	3	6	6	7	9	9
4*i*	131	127	30	82	52	288	340	185
S.e.m.	10	8	3	13	11	7	11	6
5	136	128	33	90	57	297	354	191
S.e.m.	25	8	3	4	6	30	30	6
6*o*	141	123	43	131	88	307	395	233
S.e.m.	8	7	3	8	10	12	9	13
6*i*	162	101	56	116	60	319	379	383
S.e.m.	7	3	4	6	6	9	11	12
W	229	78	81	195	114	388	502	428
S.e.m.	6	4	4	10	7	7	9	12

*S.e.m. = Standard error of the mean.

there is apparently a definite increase in the amount of myelinated fibres at layer 3*i*, as compared to the more superficial layers.

The distribution of the *cephalins and lecithins* (Tables 6 and 7) shows the expected increase of cephalins† in white matter and the slight decrease of the lecithins. Both of these lipide fractions are almost constant until the layers just superficial to the white matter are reached, when the cephalins increase rather markedly and the lecithins decrease slightly. The *sphingomyelins* are similar to the cephalins in their distribution.

Deoxyribonucleic Acid and Number of Cells (Tables 4 and 5)

The total number of cells in a given volume of the cerebral cortex has been evaluated by measuring DNA in the frozen-dried sections. In the calculation of the number of cells from the measurement of DNA, the amount of DNA per nucleus

†By the present method of analysis, the cephalin fraction includes the acetal phospholipides, which contain serine or ethanolamine.

is assumed to be constant. Evidence for this assumption is found in the study of HELLER and ELLIOTT (1954) on the cells of the cerebral cortex. DNA gives a measure of *total* number of cells; it does *not* distinguish neurons from glial cells.

In the motor cortex, it is striking that layers 2 and 3*o* have almost as few cells as has layer 1. On the other hand, white matter has more cells than layer 1 and approximately the same number of cells as the other cortical layers. Previous studies in the dog and cat (LOGAN, MANNELL, and ROSSITER, 1952; HELLER and ELLIOTT, 1954) have similarly reported an equal, or an even greater number of cells in white matter than in cortical grey matter. In the visual cortex, the molecular layer and white matter have an approximately equal number of cells. The remaining layers of the visual cortex (layers 2 to 6, inclusive) have approximately twice as many cells as have molecular layer and white matter. In layers 3*o* and 4*o* there appear to be less cells than in any other cortical layer except the molecular layer. The occurrence of relatively few cells in layer 4*o* is probably related to the fact that this is the layer in which the specific afferents (geniculocalcarine tract) terminate. An extensive axonal-dendritic plexus and the myelinated terminations of the geniculocalcarine tract occupy a greater amount of space than in adjacent layers.

In comparing motor cortex with visual cortex, the most striking difference is that visual cortex has almost twice as many cells per mm^3 as has motor cortex. There are even 50 per cent more cells in the molecular layer of visual cortex than in that of motor cortex. The subjacent white matter of the two cortices, on the other hand, has an equal number of cells.

DISCUSSION

The use of quantitative chemical methods to study the chemical and enzymatic architectonics of the cerebral cortex is feasible. However, in a tissue as intimately mixed as the cerebral cortex, the assignment of chemical and enzymatic events to a specific histologic locus is difficult. Even in a more favourable tissue for quantitative histochemistry, such as the cerebellar cortex with its clearly defined layers, this problem is a difficult one. In this study a layer-by-layer analysis of dry weight per unit volume, protein, lipide, and DNA content of the motor and visual cortices has been done. In the motor cortex this means that the distance between any two loci studied was a maximum of 500 μ, although most distances were under 400 μ. In the visual cortex, which is only half as thick as the motor cortex, the maximum distance between any two loci studied was 300 μ, and most distances were below 200 μ. Whether any changes occurred between the loci studied and therefore were overlooked will be discussed in Paper III of this series (ROBINS, SMITH, and EYDT, 1956).

The distribution of protein, dry weight, cholesterol, and total lipide in the layers of the motor cortex shows an overall decrease for protein and increase for the others in going from layer 3*o* into white matter. These constituents are relatively constant in layers 1, 2, and 3*o*. These findings are consistent with the idea that the amount of myelin progressively increases in the cortex as the subjacent white matter is approached. A more detailed analysis indicates that between layers 3*o* and 3*i* there is the first definite increase in myelin, which then continues to increase as one gets deeper into the cortex. This inferred increase of myelin in going from 3*o* to 3*i* is consistent with the myeloarchitectonic picture of the motor cortex in the monkey*

*The myeloarchitectonic pattern of monkey brain is quite similar to that of human brain (VON BONIN, 1949).

VOL
1
1956/

(see MAUSS, 1908, Plate 11), in which the most superficial heavily stained (Weigert) fibres are seen in layer 3*i*. The lighter staining myelinated fibres in layer 1 are, however, not reflected in these chemical findings. In the visual cortex, the myeloarchitectonic picture (see MAUSS, 1908, Plate 13) shows that there is an increase of Weigert stained fibres beginning at layer 3*i* and continuing into the white matter. In addition, there is a very dense network of such fibres at layer 4*o* (line of Gennari). It might be expected, therefore, that the findings in the visual cortex for these constituents would be grossly similar to those in the motor cortex, with an additional sharp change in layer 4*o*. This, however, was not found, and a different pattern for protein, total lipide, dry weight, and cholesterol is evident in visual cortex. These constituents are almost constant from layers 1 through 5, and only begin to change at layer 6*o* or 6*i*. The differences between the myeloarchitectonic patterns as seen in Weigert preparations and the results of the chemical analyses, particularly in the visual cortex, may be explained by either of at least two assumptions. First, visibility of myelin in Weigert preparations may be dependent upon the size of the sheaths rather than their total content of myelin. The presence of lipide, considered characteristic of myelin in the outer layers of both motor and visual cortices, where very few myelinated fibres can be seen with the light microscope, is consistent with there being present in these areas a considerable total volume of sheaths that are too small to be seen. On the other hand, the differences are also consistent with the assumption that the staining of myelin is dependent upon a property that is usually, but not necessarily, correlated with the chemical substances for which analyses were made. The lines of Baillarger and the deeper layers of the visual cortex with their increased amounts of stained myelin would then be examples of sites at which the unknown substance(s) responsible for staining was present in concentrations disproportionate to those of the other constituents of myelin.

Protein, lipide, and dry-weight values were similar in the motor and visual cortices, with the exception that in the inner layers of motor cortex the proportion of protein was lower, and that of lipide (except lecithins) was higher than in the inner layers of visual cortex. These findings indicate that the inner layers of the motor cortex probably contain a greater concentration of myelin than those of the visual cortex. It is, nevertheless, striking that two cortical areas so functionally different should show such similarity in the chemical findings.

In the granular layer of cerebellar cortex (ROBINS, EYDT, and SMITH, 1956) and the pyramidal layer of Ammon's horn (LOWRY, ROBERTS, LEINER, WU, FARR, and ALBERS, 1954), both of which are relatively densely packed with nerve cell bodies compared to any of the cortical layers of the cerebral cortex, there is a significantly lower lipide content than in the adjacent molecular layers in either location. This lower lipide content is most probably related to the packing of cell bodies, which are relatively low in lipide as compared with other tissues of the nervous system. In the motor and visual cortices no such decrease in lipide is found in any of the nuclear regions. In fact, the molecular cortex has as low a lipide content as any of the more cellular layers. It is probable that the total volume occupied by cells in the cerebral cortex is so small that it does not significantly affect the lipide content of the cortical layers.

The finding of a larger number of cells per mm^3 in the visual cortex than in the motor cortex is similar to the findings of SHARIFF in five other species (SHARIFF,

1953), including man. SHARIFF's figures include only neurons, whereas the present study includes both neurons and glial cells. The present data on motor cortex are similar to those of HELLER and ELLIOTT (1954) on frontal, central, and parietal cortices of man, dog, and cat. These investigators measured the number of cells in the entire depth of the cortex by another chemical method. The quantitative agreement is close in the two studies—HELLER and ELLIOTT found from 131,000 to 153,000 cells per mm^3 in the entire cortex, and our figures would average approximately 130,000 for the entire depth of the motor cortex. The percentage of these cells that are neurons cannot be decided by present chemical methods. It is of interest, however, to attempt a calculation of the numbers of neurons. POPE (1952) has reported differential counts of neurons, astrocytes, oligodendroglia, and microglia for the somatic (parietal) sensory cortex of the rat. In layers 2 through 6 the neurons constituted from 53 to 67% (mean = 58%) of the total cells; in layer 1, they constituted 21%; and in layer 6b, 40%. Although we have studied a different species and different cortical areas, if one uses POPE's figures on the present material, the number of neurons in layer 1 of motor cortex is 19,000 per mm^3, and of visual cortex 27,000; in layers 2 through 5*i* of motor cortex from 58,000 to 90,500; in layers 2 through 6*o* of visual cortex from 116,000 to 159,000; in layer 6 of motor cortex 60,000; and in layer 6*i* of visual cortex 90,000. These calculated values agree reasonably well with the value of 62,000 neurons per mm^3 given by SHARIFF for the motor cortex of *Cercopithecus*, but disagree with his value of 265,000 for the visual cortex. We find only something over 200,000 *total* cells in the visual cortex. It may be that *Cercopithecus* differs markedly from the rhesus monkey in the structure of its visual cortex, although VON BONIN and BAILEY (1947) do not believe that there is a significant difference.

ROWLAND and METTLER (1949) have recently discussed the problem of shrinkage on fixing, embedding, and staining of tissue as related to counts of cells in the cerebral cortex, pointing out that most previous studies have not taken this factor into account. In the present study, the use of a direct means for measuring this shrinkage demonstrates that enormous shrinkage of cortical tissue may occur during the histological preparation of tissue. This finding indicates that quantitative histological studies would require a careful evaluation of the amount of shrinkage, if the results are to be based on the volume of the tissue.

SUMMARY

The architectonic distribution, in the motor and visual cortices of the monkey, of dry weight per unit volume, protein, total lipide, cephalins, lecithins, sphingomyelins, total sphingolipides, non-phosphosphorus-containing sphingolipides, total phospholipides, cholesterol, and deoxyribonucleic acid (number of cells) has been determined, using quantitative microchemical methods.

The motor and visual cortices were found to present different distribution patterns of the protein and lipide constituents in the cortical layers. The motor cortex exhibited a more or less consistent decrease of protein and increase in lipides beginning at the inner portion of layer 3 and continuing into white matter, whereas the visual cortex exhibited a relatively constant level of these constituents through layer 5 and then a rapid decrease in protein and increase of lipides through layer 6 into white matter.

VOL
1
1956/

The visual cortex was shown to contain approximately twice as many total cells (neurons and glia) as the motor cortex.

REFERENCES

ALBERS R. W. and LOWRY O. H. (1955) *Analyt. Chem.* **27,** 1829.

VON BONIN G. (1942) *J. Comp. Neurol* **77,** 405.

VON BONIN G. (1949) in *The Precentral Motor Cortex*, P. C. Bucy, ed. (University of Illinois Press, Urbana), 7–82.

VON BONIN G. and BAILEY P. (1947) *The Neocortex of Macaca Mulatta* (University of Illinois Press, Urbana).

BRANTE G. (1949) *Acta Physiol. Scand.* **18,** suppl. 63.

FOLCH-PI J. (1955) in *Biochemistry of the Developing Nervous System*, H. Waelsch, ed. (Academic Press, New York), 121–132.

HELLER I. and ELLIOTT K. A. C. (1954) *Canadian J. Biochem. and Physiol.* 32, 584.

JOHNSON A. C., MCNABB A. R., and ROSSITER R. J. (1948) *Biochem. J.* **43,** 573.

LOGAN J. E., MANNELL W. A., and ROSSITER R. J. (1952) *Biochem. J.* **51,** 470.

LOWRY O. H. (1953) *J. Histochem. and Cytochem.* **1,** 420.

LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M.-L., and FARR A. L. (1954) *J. Biol. Chem.* **207,** 1.

LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU, M.-L., FARR A. L., and ALBERS R. W. (1954) *J. Biol. Chem.* **207,** 39.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L., and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.

MAUSS T. (1908) *J. f. Psychol. and Neurol.* **13,** 263.

O'LEARY J. L. (1941) *J. Comp. Neurol.* **75,** 131.

PESEZ M. (1950) *Bull. Soc. Chim. Biol.* **32,** 701.

POPE A. (1952) *J. Neurophysiol.* **15,** 115.

ROBINS E., EYDT K. M., and SMITH D. E. (1956) *J. Biol. Chem.* **220,** 677.

ROBINS E., LOWRY O. H., EYDT K. M., and MCCAMAN R. E. (1956) *J. Biol. Chem.* **220,** 661.

ROBINS E. and SMITH D. E. (1953) *Res. Publ., Ass. Res. Nerv. and Ment. Dis.* **32,** 305.

ROBINS E. and SMITH D. E. (1954) *Amer. J. Pathol.* **30,** 647.

ROBINS E., SMITH D. E., and EYDT K. M. (1956) *J. Neurochem.* **1,** 77. (This is Paper III of the present series.)

ROBINS E., SMITH D. E., EYDT K. M., and MCCAMAN, R. E. (1956) *J. Neurochem.* **1,** 68. (This is Paper II of the present series.)

ROWLAND L. P. and METTLER F. A. (1949) *J. Comp. Neurol.* **90,** 255.

SHARIFF G. A. (1953) *J. Comp. Neurol.* **98,** 381.

STOWELL R. E. (1941) *Stain Technol.* **16,** 67.

Journal of Neurochemistry, 1956, Vol. 1, pp. 68-76, Pergamon Press Ltd., London.

THE QUANTITATIVE HISTOCHEMISTRY OF THE CEREBRAL CORTEX—II

ARCHITECTONIC DISTRIBUTION OF NINE ENZYMES IN THE MOTOR AND VISUAL CORTICES*

ELI ROBINS, DAVID E. SMITH, KATHRYN M. EYDT, and RICHARD E. MCCAMAN
Department of Psychiatry and Neurology and the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri

(*Received* 11 *January* 1956)

VOL 1 1956/

IN Paper I (ROBINS, SMITH, and EYDT, 1956a) the architectonic distribution of protein, lipides, and deoxyribonucleic acid was described in the individual layers of motor and visual cortices of the monkey (*Macaca mulatta*). In this paper the distribution of nine enzymes—acid phosphatase, alkaline phosphatase, adenosinetriphosphatase (ATPase), purine nucleoside phosphorylase, glutamic dehydrogenase, fumarase, malic dehydrogenase, aldolase, and lactic dehydrogenase—in the architectonic layers of these same cortical areas is described.

MATERIAL AND METHODS

The animals used, the methods of obtaining the frozen-dried, unembedded, and unfixed cortical tissue, and the methods of defining the layers have been described in Paper I, or referred to in that paper.

The methods for measuring the enzyme activities were quantitative microchemical spectrophotometric and fluorimetric methods. These methods are described elsewhere: malic, lactic, and glutamic dehydrogenases (ROBINS, ROBERTS, EYDT, LOWRY, and SMITH, 1956); acid phosphatase, alkaline phosphatase,† ATPase,‡ aldolase, and fumarase (LOWRY, ROBERTS, WU, HIXON, and CRAWFORD, 1954); and purine nucleoside phosphorylase (ROBINS, SMITH, and MCCAMAN, 1953).

Statistical tests of significance used were *t* tests (GARRETT, 1947), in which the standard errors of the mean of each set of values were increased by the factor $\sqrt{n/n-1}$. A probability level of 0·05 or less was accepted as being statistically significant.

RESULTS

General Description of the Pattern of Distribution of Six of the Enzymes (*Tables* 1 *and* 2)

The pattern of distribution of six of the nine enzymes was remarkably similar to that of protein content, and a mirror image of that of the lipides described in Paper I. Acid phosphatase, alkaline phosphatase, ATPase, fumarase, aldolase, and lactic dehydrogenase exhibited relatively high levels of activity in layers 1 through 3*o* of the motor cortex, a slight or moderate decrease in 3*i*, and a progressive decrease throughout

* Aided by grants from the National Foundation for Infantile Paralysis, and the National Institute of Mental Health, United States Public Health Service.

† Alkaline phosphatase activity was measured using 2-amino-2-methyl-1-propanol as the buffer. Approximately twice as much activity is found with this buffer as with a glycine buffer (LOWRY, ROBERTS, WU, HIXON, and CRAWFORD, 1954).

‡ ATPase was measured in the presence of 2 mM $MgCl_2$. It was, therefore, the magnesium-activated and not the calcium-activated enzyme that was determined.

the remainder of the layers into white matter. In the visual cortex, in contrast, the activities of these enzymes remained relatively constant until layer 5*i* or one of the sublayers of layer 6 was reached, and then the activities decreased sharply into white matter. The trends in the activities of these six enzymes apparent from the analytical values are largely substantiated by the relatively frequent occurrence of statistically significant differences in activity between adjacent layers in the outer layers of the motor cortex and the infrequent occurrence of such decreases in the outer layers of the visual cortex (Tables 1 and 2). Examples of this general pattern are shown for acid phosphatase and aldolase in Figs. 1 and 2.

TABLE 1.—ACTIVITIES OF NINE ENZYMES IN THE LAYERS OF THE MOTOR CORTEX

All values expressed as moles of substrate metabolized per kilo of dry brain per hour. Each value is the mean of from nine to twenty-six measurements on three animals.

Cortical layer	Acid phospha-tase	Alkaline phospha-tase	ATPase	Purine nucleo-side phosph-orylase	Glutamic dehydro-genase	Fumar-ase	Malic dehydro-genase	Aldo-lase	Lactic dehydro-genase
1	1·92	1·56	3·55	1·58	0·686	20·9	79·8	6·34	23·8
S.e.m.†	*0·06*	*0·19*	*0·16*	*0·07*	*0·034*	*0·6*	*1·3*	*0·11*	*0·5*
2	2·07	1·46	3·35	**1·36**	0·624	**23·4***	77·4	6·27	24·6
S.e.m.	*0·08*	*0·06*	*0·17*	*0·04*	*0·027*	*0·7*	*1·3*	*0·13*	*0·5*
3*o*	1·96	**1·81***	3·39	1·32	**0·493**	22·1	**84·0***	6·27	24·1
S.e.m.	*0·06*	*0·08*	*0·19*	*0·07*	*0·031*	*1·1*	*1·2*	*0·16*	*0·5*
3*i*	**1·64**	**1·56**	**2·77**	**1·14**	**0·413**	20·1	**66·8**	**5·81**	**21·7**
S.e.m.	*0·04*	*0·07*	*0·15*	*0·03*	*0·017*	*0·6*	*0·8*	*0·16*	*0·5*
4	**1·51**	1·40	2·61	1·03	**0·352**	**18·2**	**63·3**	5·76	**19·6**
S.e.m.	*0·04*	*0·09*	*0·16*	*0·05*	*0·013*	*0·6*	*1·3*	*0·16*	*0·3*
5-*Betz*	1·47	1·21	2·68	1·05	0·347	17·8	**69·5***	6·04	**22·3***
S.e.m.	*0·04*	*0·10*	*0·17*	*0·04*	*0·018*	*0·6*	*1·4*	*0·12*	*0·6*
5*o*	**1·31**	**0·85**	2·59	1·00	0·308	17·4	**55·8**	5·73	**18·5**
S.e.m.	*0·03*	*0·05*	*0·17*	*0·03*	*0·008*	*0·6*	*1·4*	*0·11*	*0·4*
5*i*	1·18	**0·50**	2·67	0·96	**0·369***	**14·2**	52·1	**4·78**	**17·2**
S.e.m.	*0·06*	*0·04*	*0·17*	*0·03*	*0·012*	*0·5*	*1·3*	*0·17*	*0·4*
6	**0·73**	**0·36**	**1·74**	**0·84**	**0·251**	**8·6**	**33·4**	**3·58**	**14·0**
S.e.m.	*0·05*	*0·03*	*0·08*	*0·03*	*0·013*	*0·6*	*1·4*	*1·12*	*0·4*
W	**0·43**	**0·17**	**1·21**	0·83	**0·187**	**5·4**	**22·4**	**2·69**	**12·4**
S.e.m.	*0·02*	*0·02*	*0·06*	*0·04*	*0·011*	*0·9*	*0·7*	*0·10*	*0·4*

† S.e.m. = Standard error of the mean.

Values in bold face type = A statistically significant decrease in enzymatic activity when compared with the adjacent more superficial layer.

Values in bold face type* = A statistically significant increase in enzymatic activity when compared with the adjacent more superficial layer.

A probability level of 0·05 was accepted as being statistically significant.

TABLE 2.—ACTIVITIES OF NINE ENZYMES IN THE LAYERS OF THE VISUAL CORTEX

All values expressed as moles of substrate metabolized per kilo of dry brain per hour. Each value is the mean of from six to fifteen measurements on two animals. (Each value for LDH is the mean of five measurements on one animal.)

Cortical layer	Acid phospha-tase	Alkaline phospha-tase	ATPase	Purine nucleo-side phosph-orylase	Glutamic dehydro-genase	Fuma-rase	Malic dehydro-genase	Aldo-lase	Lactic dehydro-genase
1	2·20	1·79	5·26	1·74	0·601	18·8	87·0	6·56	28·8
S.e.m.†	*0·14*	*0·05*	*0·31*	*0·07*	*0·029*	*1·2*	*1·4*	*0·19*	*0·8*
2	2·07	**1·36**	4·69	1·55	**0·443**	22·3	82·7	6·18	25·9
S.e.m.	*0·09*	*0·07*	*0·16*	*0·08*	*0·018*	*1·1*	*2·0*	*0·11*	*0·5*
3*o*	1·98	1·51	4·29	1·36	**0·373**	20·4	83·1	6·44	25·3
S.e.m.	*0·10*	*0·07*	*0·33*	*0·07*	*0·020*	*0·8*	*1·1*	*0·13*	*1·4*
3*i*	1·87	1·33	4·36	1·37	0·367	19·8	78·6	6·65	26·3
S.e.m.	*0·09*	*0·05*	*0·30*	*0·06*	*0·025*	*1·1*	*3·0*	*0·16*	*0·8*
4*o*	1·95	1·46	4·30	1·55	0·326	19·6	86·6	6·54	25·2
S.e.m.	*0·07*	*0·09*	*0·34*	*0·08*	*0·012*	*0·8*	*2·3*	*0·15*	*0·6*
4*i*	1·93	1·57	3·57	**1·20**	**0·379***	19·3	83·5	6·53	25·5
S.e.m.	*0·06*	*0·08*	*0·48*	*0·08*	*0·019*	*1·2*	*4·1*	*0·20*	*0·4*
5	1·96	**1·34**	4·44	1·16	0·398	19·7	**70·6**	5·89	24·0
S.e.m.	*0·09*	*0·07*	*0·55*	*0·07*	*0·027*	*0·9*	*2·8*	*0·25*	*0·9*
6*o*	1·79	**0·76**	3·64	1·19	**0·314**	17·2	68·2	5·52	24·6
S.e.m.	*0·13*	*0·10*	*0·21*	*0·11*	*0·026*	*1·1*	*3·3*	*0·24*	*0·6*
6*i*	**0·57**	**0·22**	3·58	**0·79**	**0·146**	**13·0**	**30·5**	**3·67**	**16·9**
S.e.m.	*0·05*	*0·06*	*0·33*	*0·07*	*0·025*	*1·6*	*3·0*	*0·26*	*1·1*
W	**0·38**	0·071	**1·45**	0·62	0·100	**3·6**	**18·8**	**2·82**	**9·6**
S.e.m.	*0·03*	*0·080*	*0·08*	*0·06*	*0·007*	*0·4*	*1·1*	*0·16*	*2·0*

† S.e.m. = Standard error of the mean.

Values in bold face type = A statistically significant decrease in enzymatic activity when compared with the adjacent more superficial layer.

Values in bold face type* = A statistically significant increase in enzymatic activity when compared with the adjacent more superficial layer.

A probability level of 0·05 was accepted as being statistically significant.

Enzymes of the glycolytic cycle. Since aldolase and lactic dehydrogenase are members of the same metabolic cycle, it is not surprising (STROMINGER and LOWRY, 1955) that their activities parallel each other. Lactic dehydrogenase activity showed a slight but statistically significant peak in layer 5-*Betz* of the motor cortex, which had disappeared in layer 5*o*. There are almost no architectonic differences described between our layers 5-*Betz* and 5*o* except for the presence of Betz cells in the former. It is probable that this peak is not related to presence of Betz cells, because they are so sparsely

VOL 1 1956/

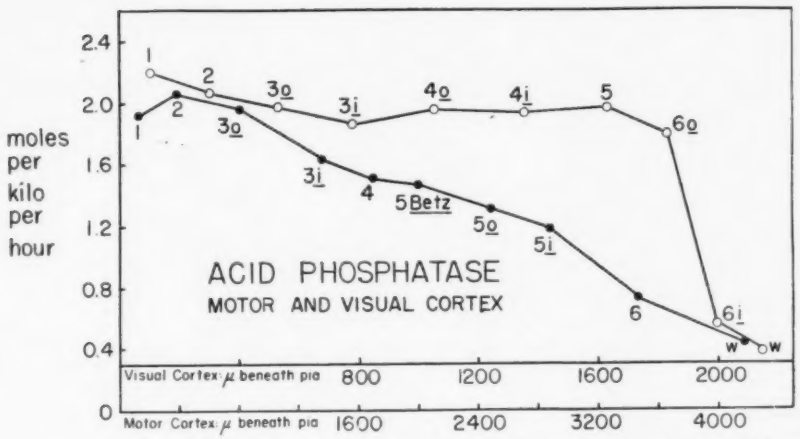


FIG. 1.—Acid phosphatase activity in the layers of the motor (●) and visual (○) cortices, showing the characteristic distribution pattern described in the text.

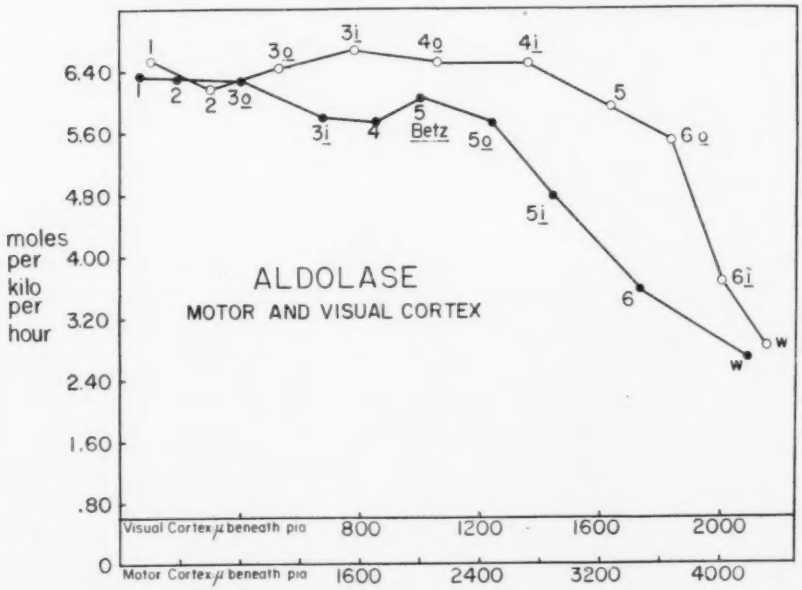


FIG. 2.—Aldolase activity in the layers of the motor (●) and visual (○) cortices, showing the characteristic distribution described in the text.

distributed and because there is no evidence to indicate that nerve-cell bodies have higher glycolytic activity than the surrounding neuropil. Aldolase also showed a slight peak in this layer, but this was not statistically significant.

Enzymes of the citric acid cycle. In the motor cortex, malic dehydrogenase activity showed a significant decrease beginning in layer 2 and continuing into white matter, with the exception of a slight but significant rise in layer 5-*Betz*. There was no corresponding increase of fumarase activity in this layer. In the visual cortex, there were no significant peaks in either malic dehydrogenase or fumarase activities. Malic dehydrogenase differs somewhat from the general pattern of visual cortex in that its activity showed a significant decrease at layer 5, rather than in layer 6*o* or 6*i* as did most of the other enzymes. It is interesting that fumarase activity was higher in layer 2 than in layer 1 in both cortices.

Phosphatases. Acid phosphatase, alkaline phosphatase, and ATPase activities followed closely the general pattern for motor and visual cortices. Alkaline phosphatase activity did show a significant peak in layer 3*o* of the motor cortex, whereas in the visual cortex its activity was highest in layer 1. It might be noted that, at least in the dog (BILLENSTIEN, 1953), there is a very dense capillary plexus in layer 5-*Betz*, which was not, in the monkey, associated with any evident increase in alkaline phosphatase activity.

Purine nucleoside phosphorylase. This enzyme exhibited an appreciably higher activity in layer 1 of both cortices, which is apparently in contrast to its distribution in the cerebellum (ROBINS, SMITH, and MCCAMAN, 1953), where it was greatest in the granular layer and only 7% greater in the molecular layer than in white matter. In the cerebral cortices, the activities in the molecular layers were 90% (motor) and 180% (visual) greater then in the corresponding white matter. These differences from cerebellum were related to both an increased activity in the molecular layers of the cerebral cortices and a decreased activity in the cerebral white matter. In the visual cortex a secondary peak of activity occurred in layer 4*o*. This suggests that perhaps the extensive terminal arborizations of the geniculocalcarine tract in this layer are associated with increased activity. This would be consistent with the finding of highest activity in the predominantly synaptic molecular layer of cerebral cortex, but inconsistent with its relatively low value for cerebellar molecular layer.

Glutamic dehydrogenase. This enzyme exhibited its highest activity in layer 1 of the cortices. This was especially striking in the visual cortex. In both cerebral cortices, the activities in the molecular layers were 263% (motor) and 501% (visual) greater than in the corresponding cerebral white matter. As in the case of purine nucleoside phosphorylase, these results are unexpected when compared to those for the cerebellum (ROBINS, ROBERTS, EYDT, LOWRY, and SMITH, 1956), where glutamic dehydrogenase activity was only 39% greater in molecular cortex than in white matter. However, the molecular layer of Ammon's horn in the rabbit is richer in glutamic dehydrogenase than any of the other layers (STROMINGER and LOWRY, 1955). The glutamic dehydrogenase activity decreased from layer I until layer 5*i* of the motor cortex was reached, where it rose and peaked (Table 1, and see Fig. 3 of Paper III (ROBINS, SMITH, and EYDT, 1956b)). In the visual cortex, the activity decreased until layer 4*i* was reached, where it began to rise, reaching a peak in layer 5, and not returning to its level in layer 4*o* (just before the increase began) until later 6*o* was reached (Table 2, and see Fig. 6 of Paper III). Layer 5*i* of the motor cortex and layer 5 of the visual cortex are both

VOL
1
1956/

relatively sparsely populated with small pyramidal cells (in stained sections, but see Paper I for total cell counts) and contain a plexus formed by basal dendrites of these cells and axons of the inner line of Baillarger. Whether there is something special about these cells or plexuses that gives rise to increased glutamic dehydrogenase activity, is not known. By measuring various enzymes concerned with glutamate metabolism and by measuring glutamate and related compounds, it should be possible to determine whether this increased glutamic dehydrogenase activity is specific for this enzyme or related to glutamate metabolism generally.

Comparison of Levels of Enzymatic Activities in the Motor and Visual Cortices

Less difference was found in the absolute levels of enzymatic activities between the motor cortex and the visual cortex than expected in view of the considerable functional and cytoarchitectonic differences between the two cortices.* The inner cortical layers proper (excluding white matter) of visual cortex did tend to have higher enzymatic activities than the inner layers of motor cortex, which was a reflection of the general pattern above described. This was analogous to the findings for protein and lipide described in Paper I of this series.

The only enzyme that showed a marked difference between the cortical areas was ATPase, which was 33% richer in visual than in motor cortex, even in the more superficial layers. This difference was even greater in the deeper layers, until white matter was reached, when the activities became about equal in the two locations. The finding of a higher ATPase activity in visual cortex generally and the higher enzymatic activities for the other enzymes in the inner layers suggests perhaps that there is more total energy metabolism in the visual cortex than in the motor cortex (LOWRY, ROBERTS, LEINER, WU, FARR, and ALBERS, 1954).

The subjacent white matter of the visual cortex was appreciably less rich in glutamic dehydrogenase and alkaline phosphatase than was the subjacent white matter of the motor cortex.

DISCUSSION

The contrast between the motor cortex and visual cortex in the overall distribution patterns of six of the nine enzymes studied, of protein content, and of lipide content, is striking. In the motor cortex, enzyme activities are high in layers 1 through 3*o*, decrease in layer 3*i*, and continue to decrease into white matter; in the visual cortex, the enzymatic activities tend to be relatively constant through layers 5 or 6*o* and decrease rather abruptly into white matter. Protein content follows this pattern, and lipides are a mirror image of it. A possible histologic correlate of this difference may be the size and packing of the myelinated fibres in the deeper layers. In the motor cortex a vertical (radial) columnization is seen from layer 3*i* inwards, which is caused by the myelinated fibres entering and leaving the cortex. In the more cellular visual cortex, this columnization has virtually disappeared (VON BONIN, 1950). It is probable that, in the visual cortex, the myelinated fibres are slenderer than in the motor cortex and constitute a smaller proportion of the total volume than they do in the motor cortex.

* It might also be pointed out that there are marked differences in the susceptibility of these areas to various pathologic processes. For example, the motor cortex is often severely affected by the poliomyelitis virus, whereas the visual cortex is almost completely resistant (BODIAN and HOWE, 1941; BAKER, CORNWELL, and TICHY, 1954).

All of the nine enzymes studied were richest in the outer layers of both cortices.* In the motor cortex, four of the nine enzymes were richest in layer 1 and the remaining five in layers 2 or 3*o*. In the visual cortex, seven of the nine enzymes were richest in layer 1, one was richest in layer 2, and one in layer 3*i*. These results are not simply a reflection of the greatly increased amount of myelin (lipide) in the deepest layers or subjacent white matter. When the activities are recalculated, using protein content rather than dry weight as a base, the outer layers of both cortices are still the richest. In both cortices, when the results are based on protein content, only aldolase activity peaks in an inner layer—layer 5-*Betz* in the motor cortex and layer 4*i* in the visual cortex. (Lactic dehydrogenase peaks at layer 4*o* in the visual cortex). The greater enzymatic activities in the outer layers of both cortices are related, therefore, to other structural elements than simply to the relative amounts of myelin in different layers. It is probable that this difference is related to the bulk of dendritic cytoplasm, since "brain metabolism" is quantitatively chiefly "dendrite metabolism" (LOWRY, ROBERTS, LEINER, WU, FARR, and ALBERS, 1954). This would indicate that the proportion of the total volume occupied by dendritic material is greater in the plexuses of the outer layers than in those of the inner layers, even when the amount of lipide is excluded by basing the results on protein content.

The relatively slight differences in enzymatic activities between layers 1 and 2 in both cortices is noteworthy. In the motor cortex only purine nucleoside phosphorylase (14% lower in layer 2) and fumarase (12% higher) showed a greater than 10% difference in the two layers. In the visual cortex, four enzymes—alkaline phosphatase (24% lower in layer 2), purine nucleoside phosphorylase (11% lower), glutamic dehydrogenase (26% lower), and fumarase (18% higher)—showed more than a 10% difference. These rather small differences between two layers which appear quite distinct histologically, are probably accounted for by there being an approximately equal content of dendritic material in the two layers. This suggests that the degree of arborization of dendritic plexuses, which is presumably more in layer 1 than in layer 2, is less important than the total amount of dendritic material present.

DIXON (1953) has presented data on overall glycolytic rates measured manometrically in the rabbit cerebral cortex (superior surface). He contrasted the glycolytic activity of the molecular cortex with the deeper layers of cortex (excluding white matter) and found a ratio of activity of approximately 1·7:1 based on dry weight (1·4:1 based on wet weight). These findings are consistent with the present findings for aldolase and lactic dehydrogenase in the motor cortex. These enzymes were richer in the outer layers than in the inner layers. In the visual cortex, which DIXON did not study, a more nearly equal glycolytic rate would be expected when molecular cortex was compared with the deeper cortical layers.

The four enzymes of the glycolytic and citric-acid cycles bear a surprisingly constant relationship to one another throughout the depth of each cortex until layer 6 is reached. The ratios of malic dehydrogenase to lactic dehydrogenase activity and of fumarase to aldolase activity range from approximately 3 to 3·5 in both cortices, until layer 6 of the motor cortex and layer 6*i* of the visual cortex is reached. In the motor cortex, in layer 6 the ratios decrease to 2·4, and in white matter to 2·0 or less.

* There was a greater difference in enzymatic activities between the outer and inner layers of the motor cortex than between the corresponding layers of visual cortex. This is expected, since, as has been indicated, the activities throughout the depth of the cortex show less decrease for visual cortex than for motor cortex.

VOL
1
1956/

In the visual cortex, in layer 6*i* the malic dehydrogenase/lactic dehydrogenase ratio falls to 1·8, and in white matter both this ratio and the fumarase/aldolase ratio are 2·0 or less. The constancy of these ratios from layers 1 through 5 suggests that the *relative* activities of the glycolytic and citric acid cycles in the cerebral cortex are largely independent of the histologic composition of the individual layers. This is a surprising finding, since it implies that in the cerebral cortex these two important metabolic cycles do not change in their activities relative to each other in cortical dendrites, axonal telodendria, or axons. There is some evidence, at least for the granular cells of the cerebellum, that aldolase activity is disproportionately low in the cell bodies (ROBINS and SMITH, 1953). The cell bodies of the cerebral cortex are too sparsely distributed to settle this point for the nerve cell bodies of the cerebral cortex.

The decrease of these ratios to approximately 2·0 in white matter indicates that glycolysis, when contrasted with the citric acid cycle, assumes a relatively more important role in white matter than it has in grey matter. These findings have also been found in the cerebellar cortex and its subjacent white matter (ROBINS and SMITH, 1953; ROBINS, ROBERTS, EYDT, LOWRY, and SMITH, 1956).

It is of interest to compare the ratios of activity in white matter to that in the molecular layer for all these enzymes in the two cerebral cortices. When this is done, the enzymes may be classified into two groups. One group—acid phosphatase, alkaline phosphatase, fumarase and malic dehydrogenase—shows a decrease of activity in white matter to less than 30% (4 to 28%) of that in layer 1 in both cerebral cortices and in the cerebellar cortex. The other group—purine nucleoside phosphorylase, aldolase, and lactic dehydrogenase—shows a lesser decrease (31 to 93% of their values in layer 1) in white matter. This division suggests that the latter group of enzymes has a specialized function and location in white matter. It is likely that they are relatively rich in oligodendroglia, axis cylinders, and/or the protein moiety of myelin itself. ATPase is borderline in that its activity decreases almost exactly 30% (28 to 34%) in the three locations. Glutamic dehydrogenase is anomalous. Its activity decreases to 27% and 17 % in the white matter subjacent to the motor and visual cortices respectively, whereas in cerebellar cortex it decreases only slightly to 72% of its value in the cerebellar molecular layer (ROBINS, ROBERTS, EYDT, LOWRY, and SMITH, 1956). This contrast between the distribution of glutamic dehydrogenase in the cerebral cortex and cerebellar cortex is chiefly caused by a very low glutamic dehydrogenase activity in the cerebellar molecular layer.

The decrease in ATPase activity in white matter to 34 and 28% of its value in layer 1 of the motor and visual cortices respectively, is somewhat different from the decrease in white matter to only 75% of its value in layer 1, found by HESS and POPE (1954) in the rat somatosensory cortex. These investigators have also shown that there are peaks of activity, varying from 7 to 15% higher than the adjacent measurements,* for magnesium-activated ATPase at the junctions of layers I–II, at layer III*b* and at layer V*c*.

The activities of the nine enzymes studied were very similar in the motor and visual cortices. It is probable that the susceptibility of these cortices to disease processes which affect the two cortices differentially, for example poliomyelitis, does not reside in disturbances in these particular enzymes. If the glycolytic and citric acid

* These percentages were calculated by the present authors from a chart presented by POPE (1955).

cycle enzymes studied may be taken as representative of these metabolic cycles, it is possible to go a step further and speculate that glycolysis and citric acid cycle metabolism are not primarily involved in such disease processes.* This conclusion is consistent with the findings of WOOD, RUSOFF, and REINER (1945), in which they reported normal anaerobic glycolysis in poliomyelitis-infected brain.

SUMMARY

The architectonic distribution in the motor and visual cortices of the monkey of acid phosphatase, alkaline phosphatase, ATPase, purine nucleoside phosphorylase, glutamic dehydrogenase, fumarase, malic dehydrogenase, aldolase, and lactic dehydrogenase has been determined, using quantitative microchemical methods.

The motor and visual cortices were found to present a different pattern of distribution for six of these enzymes in the cortical layers. Purine nucleoside phosphorylase and glutamic dehydrogenase exhibited a different pattern in both cortices from the remainder of the enzymes.

The absolute levels of activities of these enzymes were similar, except for ATPase, in the motor and visual cortices, although, in general, there was a somewhat higher level of enzymatic activity in the inner layers of visual cortex as compared with those in the motor cortex. This latter finding, coupled with the finding of higher ATPase activity in the visual cortex, suggests that total energy metabolism is greater in the visual cortex than in the motor cortex.

REFERENCES

BAKER A. B., CORNWELL S., and TICHY F. (1954) *Amer. Med. Ass. Arch. Neurol. and Psychiat.* **71,** 435.

BILLENSTIEN D. C. (1953) *Anat. Rec.* **117,** 129.

BODIAN D. and HOWE H. A. (1941) *Bull. Johns Hopkins Hosp.* **68,** 58.

VON BONIN G. (1950) *Essay on the Cerebral Cortex* (Charles C. Thomas, Springfield, Ill.).

DIXON K. C. (1953) *J. Physiol.* **120,** 267.

GARRETT H. E. (1947) *Statistics in Psychology and Education, 3rd ed.* (Longmans, Green and Co., New York).

HESS H. H. and POPE A. (1954) *Federation Proc.* **13,** 228.

LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M.-L., FARR A. L., and Albers R. W. (1954) *J. Biol. Chem.* **207,** 39.

LOWRY O. H., ROBERTS N. R., WU M.-L., HIXON W. S., and CRAWFORD E. J. (1954) *J. Biol. Chem.* **207,** 19.

POPE A. (1955) *J. Neuropathol. and Exper. Neurol.* **14,** 39.

ROBINS E., ROBERTS N. R., EYDT K. M., LOWRY O. H., and SMITH D. E. (1956) *J. Biol. Chem.* **218,** 897.

ROBINS E. and SMITH D. E. (1953) *Res. Publ., Assn. Res. Nerv and Ment. Dis.* **32,** 305.

ROBINS E., SMITH D. E., and EYDT K. M. (1956a) *J. Neurochem.*, **154** (This is Paper I of the present series).

ROBINS E., SMITH D. E., and EYDT K. M. (1956b) *J. Neurochem.*, **177** (This is paper III of the present series).

ROBINS E., SMITH D. E., and MCCAMAN R. E. (1953) *J. Biol. Chem.* **204,** 927.

STROMINGER J. L. and LOWRY O. H. (1955) *J. Biol. Chem.* **213,** 635.

WOOD H. G., RUSOFF I. I., and REINER J. M. (1945) *J. Exp. Med.* **81,** 151.

* It should be pointed out, however, that nerve-cell bodies are primarily involved in poliomyelitis, and that differences in them may be obscured by the presence of the relatively large amounts of dendritic material in both cortices.

Journal of Neurochemistry, 1956, Vol. 1, pp. 77-83, Pergamon Press Ltd., London.

THE QUANTITATIVE HISTOCHEMISTRY OF THE CEREBRAL CORTEX—III

ANALYSES AT 50 μ INTERVALS COMPARED WITH ANALYSES BY ARCHITECTONIC LAYERS IN THE MOTOR AND VISUAL CORTICES*

ELI ROBINS, DAVID E. SMITH, and KATHRYN M. EYDT
Department of Psychiatry and Neurology and the Department of Pathology,
Washington University School of Medicine, St. Louis, Missouri

VOL.
1
56/57

(*Received* 11 *January* 1956)

THE measurements of chemical constituents and enzymes in the motor and visual cortices of the monkey reported in the first two papers of this series (ROBINS, SMITH, and EYDT, 1956; ROBINS, SMITH, EYDT, and MCCAMAN, 1956) were made at nine architectonic levels of the cortex and in the immediately subjacent white matter. In the motor cortex, which is approximately 4 mm thick from pial surface to subjacent white matter, this means that each measurement averaged approximately 400 μ apart. In the visual cortex, which is approximately 2 mm in thickness, this means that each measurement averaged approximately 200 μ apart. Whether changes in the amounts of any of these constituents occurred between the loci measured was an important question. It was decided to measure protein, fumarase, and glutamic dehydrogenase at 50 μ intervals throughout the depth of the cortex. Protein was chosen as an example of a chemical constituent, fumarase as an enzyme with a distribution in both cortices similar to the majority of the other enzymes studied, and glutamic dehydrogenase as an enzyme with an atypical distribution. Also, it was of interest to confirm the previously found increase in glutamic dehydrogenase activity in the deeper layers of both cortices.

MATERIAL AND METHODS

Animals used were adult monkeys (*Macaca mulatta*). There was one animal used for the 50 μ series in the motor cortex and a second animal for the 50 μ series in the visual cortex. Unavoidably, in the motor cortex, it was not possible to use one of the same monkeys for the 50 μ series that had been used for the determinations on the individual architectonic layers; in the visual cortex, the monkey used for the 50 μ series was one of the two monkeys used for the determinations on the individual architectonic layers.

The tissue for the 50 μ series was obtained as described in Paper I. Cylinders were sectioned at 25 μ and alternate sections were used for analyses. From these alternate sections, five pieces of tissue weighing 1 to 3 μg, dry weight, were dissected for microchemical analysis by methods described elsewhere (LOWRY, ROSEBROUGH, FARR, and RANDALL, 1951; LOWRY, ROBERTS, WU, HIXON, and CRAWFORD 1954; ROBINS, ROBERTS, EYDT, LOWRY and SMITH, 1956). A total of nearly 2000 individual analyses were done in order to obtain measurements of protein and the two enzymes at eighty-seven levels in the motor cortex and forty-five levels in the visual cortex.

*Aided by grants from the National Foundation for Infantile Paralysis and the National Institute of Mental Health, United States Public Health Service.

RESULTS

Motor Cortex. The distribution pattern* of *protein* in the 50 μ series was very similar to that in the layer series (Fig. 1). In both, protein was highest in layers 1, 2, and 3*o*; there was a decrease in 3*i*; a slight increase in layer 4 (layer series) or at the junction of layers 4–5-*Betz* (100 μ deeper in the 50 μ series); and then a gradual parallel decrease in both from layer 5-*Betz* into white matter. From the 50 μ series

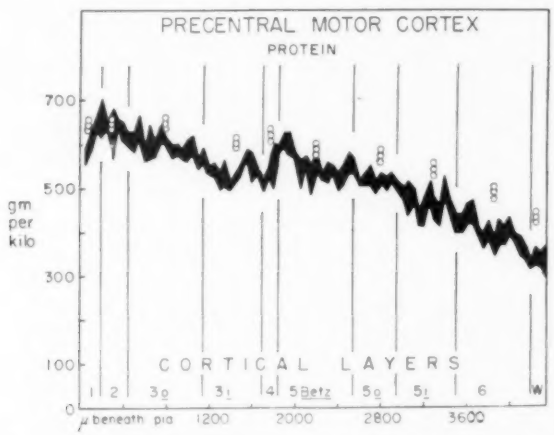


FIG. 1.—Protein content of motor cortex. The middle open circle (o) of the three in each architectonic layer is the mean, the upper one is *plus* one standard error of the mean, and the lower one is *minus* one standard error of the mean for data presented in Table 4, Paper I. The blacked-in curve was plotted from data obtained, as described under Method, at 50 μ intervals (five measurements at each interval) throughout the depth of the cortex and the immediately subjacent white matter. The position and limits of this curve were defined by the mean $\pm$ one standard error of the mean in a manner identical with that described for the open circles.

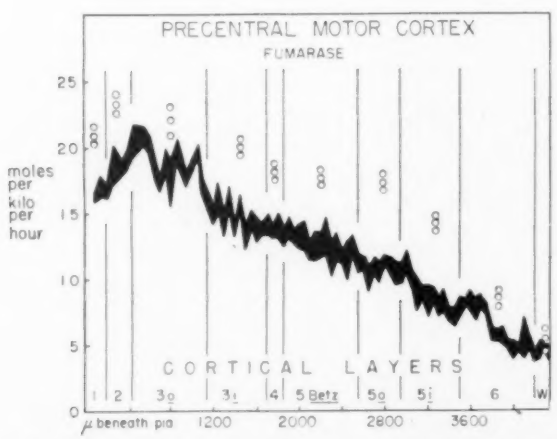


FIG. 2.—Fumarase of motor cortex. Data at each architectonic layer (o) from Table 1, Paper II. Explanation of curves as in Fig. 1.

*In the motor cortex, although the distribution patterns will be shown to be similar, the absolute levels differed to some extent in the 50 μ series and the layer series. This difference in absolute levels was most marked for fumarase. Presumably this difference in levels resulted from the necessity of having to use a different animal for the 50 μ series (see Material and Methods) in the motor cortex.

VOL 1 1956/

additional information was obtained about the following: (a) The decrease in going from 3*o* to 3*i* began in the inner part of 3*o* and continued into 3*i*. (b) No significant peaks or troughs of protein content were overlooked in the layer series.

The distribution pattern of *fumarase* in the 50 μ series was also very similar to that in the layer series (Fig. 2), even through the absolute levels of activity were different in the two series (see footnote on p. 78). In both series, fumarase activity was slightly lower in layer 1 than in layer 2; in the layer series a peak was reached in layer 2, whereas in the 50 μ series a peak was reached at the junction of layers 2–3*o* (100 μ deeper); and in both there was an almost parallel decrease from layer 3*o* into white

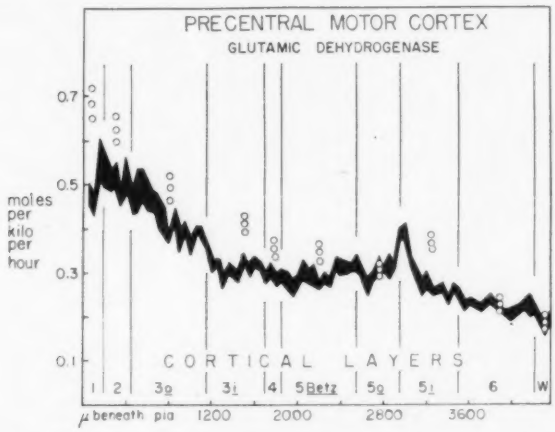


FIG. 3.—Glutamic dehydrogenase of motor cortex. Data at each architectonic layer (o) from Table 1, Paper II. Explanation of curves as in Fig. 1.

matter. From the 50 μ series additional information was obtained about the following: (a) The increase in fumarase activity in going from layer 1 to the junction of layers 2–3*o* was gradual and almost linear. (b) The decrease in going from layers 3*o* to 3*i* began at the junction of these layers, although a slight decrease had already occurred in layer 3*o* when compared with the peak at the junction of layers 2–3*o*. (c) The inner portion of layer 6 exhibited an appreciably lower fumarase activity than the outer portion. (d) No significant changes in activity in the layer series were overlooked, although the locations at which these changes began or ended could be more precisely defined.

The distribution pattern of *glutamic dehydrogenase* in the 50 μ series was very similar to that in the layer series (Fig. 3). There were two levels where possible discrepancies existed, but these were not major ones. In the 50 μ series the enzymatic activity was low at 50 and 100 μ below the pial surface and then reached a peak at 150 μ; in the layer series, layer 1 was the locus of highest activity, giving no suggestion of a lower activity immediately below the pial surface. The rise of activity in layer 5*i* of the layer series was duplicated by a rise in the outer part of layer 5*i* in the 50 μ series, although this latter rise was at a cortical depth approximately 200 μ more superficial than the former. It may be concluded that no significant changes in glutamic dehydrogenase content were overlooked in the layer series.

Visual Cortex. The distribution pattern of *protein* in the 50 μ series was very

similar to that in the layer series (Fig. 4). There was a very slight rise in the 50 μ series in layer 3*o* which was not reflected in the layer series. Otherwise, no appreciable changes were overlooked in the layer series.

The distribution pattern of *fumarase* in the 50 μ series was also similar to that in

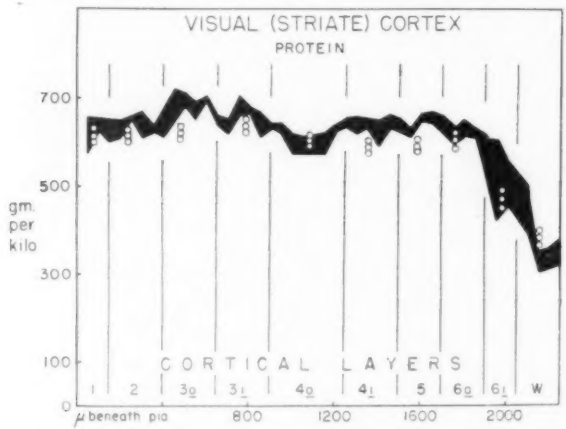


FIG. 4.—Protein content of visual cortex. Data at each architectonic layer (o) from Table 5, Paper I. Explanation of curves as in Fig. 1.

the layer series (Fig. 5). In the layer series, there was perhaps a slightly greater rise in layer 2 and a lesser decrease in layer 3*i* and layer 5. These differences, however, were only in the order of 5 to not more than 12%. The 50 μ series indicates the peak in

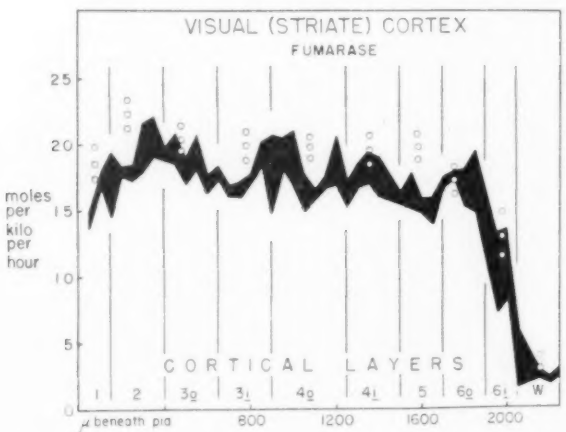


FIG. 5.—Fumarase of visual cortex. Data at each architectonic layer (o) from Table 2, Paper II. Explanation of curves as in Fig 1.

layer 2 occurs in its inner portion and it also suggests than the outer portion of layer 3*i* shows a trough in activity which has disappeared in the inner part of layer 3*i*.

The distribution pattern of *glutamic dehydrogenase* in the 50 μ series was almost identical with that in the layer series (Fig. 6). This confirmed the rise beginning in

layer 4*i*, reaching a peak in layer 5, and the beginning decrease in layer 6*o*. The only discrepancy in the two series was a greater apparent decrease of enzymatic activity in layer 6*i* of the layer series than in the 50 μ series. However, even in this case, only 50 to 75 μ deeper in layer 6*i* there was an equivalent decrease in the 50 μ series. From the 50 μ series a more precise localization of these changes in activity could be made: (a) The decrease in going from layer 1 to 2 began in layer 1. (b) The increase in layers 4*i* and 5 began at the junction of layers 4*o*– 4*i*. (c) The decrease in layer 6*i* occurred chiefly in its inner portion. In spite of this increased precision in the 50 μ series, no significant changes had been overlooked in the layer series.

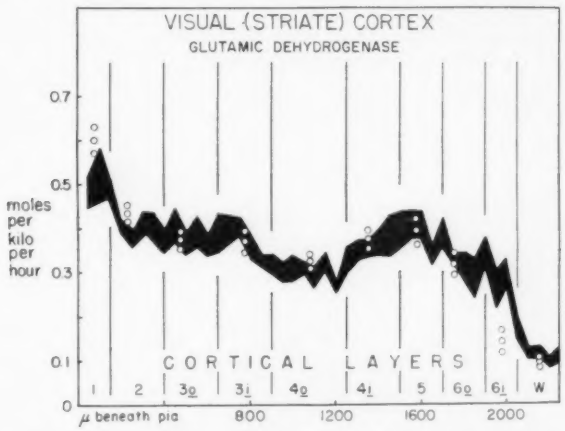


FIG. 6.—Glutamic dehydrogenase of visual cortex. Data at each architectonic layer (o) from Table 2, Paper II. Explanation of curves as in Fig. 1.

General description of the 50 μ *series* (Figs. 1 to 6). It was striking that in these six series, which comprised measurements at eighty-seven levels for each constituent in the motor cortex and measurements at forty-five levels for each constituent in the visual cortex, there were few even moderately abrupt changes from one 50 μ locus to the next. There were only thirteen changes greater than 15% in the 261 measurements in motor cortex. None was greater than 23%. There were eighteen changes greater than 15% in the 135 measurements in visual cortex. Only two (44 and 37%) were greater than 21%. These changes in the visual cortex were mostly confined in the the narrow depth between 6*o* and white matter, where these abrupt changes had already been noted in the layer series. These data on the deepest layers of the visual cortex suggest that, where there is a marked change in histology, abrupt chemical changes may occur, i.e. the sharp demarcation between 6*i* and white matter in the visual cortex.

DISCUSSION

The present study was undertaken to supplement the previous analyses of the architectonic layers of the monkey motor and visual cortices by analysing samples at 50 μ intervals throughout the depth of the cortices, in order that significant changes in protein content and enzymatic activity were not overlooked. This was especially important in view of the studies of POPE and his associates (POPE, 1952; POPE, 1955) which have shown significant changes in the activities of cytochrome oxidase, acetylcholinesterase, two kinds of ATPase, and dipeptidase at various junctional sites in the

rat somatosensory cortex. In the present study at 50 μ intervals no such abrupt changes at junctional sites were found for protein content, fumarase activity, or glutamic dehydrogenase activity, which had not been indicated in the analyses by architectonic layers.

In the motor and visual cortices, analyses by architectonic layers at ten levels gave virtually the same information as analyses at 50 μ intervals—eighty-seven levels for motor cortex and forty-five levels for visual cortex—for protein, fumarase, and glutamic dehydrogenase. These results cannot be stated to apply to any other constituent unless it is actually experimentally tested. However, the present results do suggest that as a screening procedure a layer analysis is sufficient, or in a thick cortex such as motor cortex an analysis at 200 μ intervals in addition to a layer analysis is sufficient. Where there are marked histological differences with well-segregated layers such as in the cerebellar cortex, then these well-defined layers, no matter what their thickness, should be analysed separately. In these well-segregated layers abrupt changes are found (ROBINS and SMITH, 1953).

In the interests of economy in studying such a complex structure as the cerebral cortex, it may be advantageous to measure approximately ten levels throughout the cortex and/or to concentrate on any segregated or markedly different histological layers. This procedure will sacrifice some precision, but may lead to a more rapid survey of various cortical areas. As has been indicated under Results, the chief gain in precision in this study has been to localize more accurately where the changes begin and end. On the other hand, no important changes were found in the 50 μ series that had not been found in the layer series.

Where there are abrupt and relatively slight (in terms of per cent change) but significant changes, as POPE (1952) has found for acetylcholinesterase in layers II and III of the rat somatosensory cortex, then analyses by architectonic layers and sublayers will be found wanting. POPE found a high cholinesterase activity at the junction of layers II–IIIa and in layer IIIb. Using only a layer analysis, one would probably have overlooked these changes (approximately 15%). On the other hand, the remainder of the peaks for acetylcholinesterase activity described by POPE (1952) would have been found in a layer series.

Finally, in view of the paucity of any detailed quantitative histologic studies of the cerebral cortex, more rapid progress in the future may be made by utilizing more sensitive microchemical methods, combined with the use of microdissection, to analyse individual histologic components such as Betz cells or apical dendrites.

SUMMARY

In the motor and visual cortices, analyses at 50 μ intervals (eighty-seven levels in motor cortex, forty-five in visual cortex) for protein, fumarase, and glutamic dehydrogenase revealed no striking differences in the distributions of these constituents from those found by analyses at ten loci, one at each architectonic layer, in the two cortices. It is suggested that in an intimately mixed area such as the cerebral cortex, it may be more economical to confine first studies to a layer-by-layer analysis than to do an extensive consecutive series at 50 μ or 100 μ intervals. This procedure may sacrifice some precision in localization, but the chances of overlooking important changes seem small.

VOL
1
1956/

REFERENCES

LOWRY O. H., ROBERTS N. R., WU M.-L., HIXON W. S., and CRAWFORD E. J. (1954) *J. Biol. Chem.* **207,** 19.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L., and RANDALL R. J. (1951) *J. Biol. Chem.* **193,** 265.

POPE A. (1952) *J. Neurophysiol.* **15,** 115.

POPE A. (1955) *J. Neuropathol. and Exper. Neurol.* **14,** 39.

ROBINS E., ROBERTS N. R., EYDT K. M., LOWRY O. H., and SMITH D. E. (1956) *J. Biol. Chem.* **218,** 897.

ROBINS E. and SMITH D. E. (1953) *Res. Publ., Assn. Res. Nerv. and Ment. Dis.* **32,** 305.

ROBINS E., SMITH D. E., and EYDT K. M. (1956) *J. Neurochem.* **1,** 54. (This is Paper I of the present series.)

ROBINS E., SMITH D. E., EYDT K. M., and MCCAMAN R. E. (1956) *J. Neurochem.* **1,** 68. (This is Paper II of the present series.)

VOL.
1
66/57

Journel of Neurochemistry, 1956, Vol. 1, pp. 84-90, Pergamon Press Ltd., London.

ENZYMATIC STUDIES OF MITOCHONDRIA AND OTHER CONSTITUENTS OF LOBSTER AND SQUID NERVE FIBRES*

JOHN M. FOSTER
Biology Department, Massachusetts Institute of Technology,
Cambridge, Massachusetts

(*Received* 2 *February* 1956)

VOL.
1
1956/

BEFORE it is possible to understand the energy coupling which underlies impulse propagation in peripheral nerve fibres, it is necessary to obtain information concerning not only the molecular and macromolecular structure of the nerve fibre (the structure of the machine), but also the source of the chemical energy. In this connection ABOOD and GERARD (1953) studied the particulates isolated from mammalian nerve, and to show that passage of current through suspensions of nerve mitochondria inhibited the phosphorylation of the mitochondria. MULLINS (1953) found that electrical stimulation inhibits the uptake of $P^{32}O_4$ by frog sciatic-nerve fibres. It has also been shown by KOECHLIN (1950) and others (LEWIS, 1952; SCHMITT, BEAR, and SILBER, 1939; SILBER, 1941; SILBER and SCHMITT, 1940) that dicarboxylic acids of the KREBS cycle, such as malic, fumaric, and succinic, and compounds related to them metabolically, constitute an appreciable fraction of the total anions in invertebrate nerve.

Since the general programme of research on nerve chemistry in this laboratory concerns primarily invertebrate nerves such as the giant fibre of the squid and peripheral nerves of lobster, it was desirable to determine whether mitochondria isolated from these fibre types behave in typical fashion chemically.

Electron microscope investigations of the structure of lobster fibres in thin sections show not only that the mitochondria are clustered almost exclusively at the periphery of the axon just under the Schwann cell-axon interface, but also that the Schwann cell itself appears to form mitochondria-like particulates by evaginations from the axon surface of the Schwann cell (GEREN and SCHMITT, 1954). Such observations, together with other facts, suggest that the SCHWANN cell may play a significant role in the chemical metabolism of the fibre and possibly in the energy coupling which underlies the process of impulse propagation itself. Using conventional extrusion methods, LIBET (1948) found the ATP-ase activity of the sheath to be about a hundred times higher than the axoplasm. Choline esterase is also said to be concentrated in the sheath (NACHMANSOHN, 1952). To get information about the chemical properties of the Schwann cell, new methods were developed which make possible the preparation of the squid giant fibre sheath with a minimum of damage.

* These studies were aided by a grant (NR 119–100) from the Office of Naval Research, Department of the Navy, grant B 24 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, U.S. Public Health Service, and a grant from the Trustees under the wills of CHARLES A. KING and MARJORIE KING to the Massachusetts Institute of Technology.

MATERIALS AND METHODS

Isolation of Lobster Nerve Mitochondria

The walking legs and claws of fresh live Maine hardshell lobsters were removed with clippers and the nerves rapidly removed, blotted, and stored in a chilled beaker. They were then weighed, minced thoroughly with scissors, and homogenized in ice-cold 0·25-M sucrose solution. Unbroken cells, nuclei, and other debris were removed by three ten-minute centrifugations at 600 g. The mitochondria were then sedimented from the supernatant by centrifugation in the Spinco preparative ultracentrifuge for 20 min at 15,000 g, following in general the directions of UMBREIT *et al.* (1949). The sediments were combined in a volume of 1 to 2ml sucrose solution, transferred to a smaller tube, and recentrifuged 20 min at 15,000 g. This left a clear supernate. The final sediment was resuspended with a small nylon homogenizer in an appropriate volume of sucrose solution for use in experiments.

Isolation of Mitochondria from Squid Axoplasm

Squid axoplasm presents a special problem because of its high concentration of filamentous protein (MAXFIELD, 1953) which greatly increases its viscosity. Consequently, a different procedure was employed. The axoplasm from twenty to forty squid was taken up in 1 ml 0·25-M sucrose solution and centrifuged for 45 min at 140,000 g. This sedimented the mitochondria and a considerable fraction of the filaments. The sediment was resuspended in 0·5 ml 0·25-M sucrose solution and carefully layered over 0·5 ml 0·88-M sucrose solution in another tube. It was then centrifuged 30 min at 80,000 g, which, although reducing the yield, gave a mitochondrial preparation free of filaments.

Chemical and Enzymatic Analyses

Succinic dehydrogenase was determined by the method of COOPERSTEIN, LAZAROW, and KURFESS (1950), cytochrome oxidase according to COOPERSTEIN and LAZAROW (1951), except that it was necessary to use ten times the recommended amount of "tissue." Oxidative phosphorylation was studied in constant-volume differential respirometers equipped with 5-ml flasks. At the end of the incubation period the solution was deproteinized with an equal volume of 1 N $HClO_4$ and analysed immediately for inorganic phosphate by a modification (SIEKEVITZ and POTTER, 1953) of the method of LOWRY and LOPEZ (1946). Aliquots of the tissue preparations were taken for protein analysis by the method of LOWRY *et al.* (1951). Samples of the mitochondrial preparations were also taken for electron microscopy. For this purpose the mitochondria were isolated from 0·88 M sucrose solution, by sedimenting them at 24,000 g.

Preparation of Isolated Squid Giant Fibre Sheath

Previous studies by others on isolated "sheath" preparations have usually been done on the residue remaining after extrusion of the axoplasm from the fibre. However, such a procedure might damage the Schwann cells considerably, resulting in misleading conclusions regarding the extent to which the sheath takes part in the chemical metabolism of the fibre. Consequently, a different, more gentle, procedure was adopted. The squid nerve was placed in a dish of cold sea-water under slight tension and cleaned of small fibres and connective tissue. One point of a pair of fine iridectomy scissors was then inserted into the fibre while it was still under tension, and the sheath was carefully cut lengthwise, the process being observed through a binocular microscope. The axoplasm could then be washed away with fresh sea-water. Electron micrographs of this preparation (kindly made by Dr. B. B. GEREN) showed that the Schwann cells and their axonal membranes had suffered surprisingly little damage.

Respirometry

Oxygen consumption by whole fibres and separated sheaths was measured in Scholander microvolumeters at 15°C (SCHOLANDER *et al.*, 1952). For this purpose the fibres were usually cut in half (after double ligation to prevent loss of axoplasm), one half providing the separated sheath preparation while the other was left intact to serve as a control. Each half-length was fastened to a fine glass rod for ease of handling. At the conclusion of the experiment the material was treated briefly with 10% trichloroacetic acid, dried, and dissolved in a known volume of 2-N NaOH for protein analysis.

RESULTS

In any quantitative study of the localization and distribution of enzymes in cellular fractions, certain criteria should be met (UMBREIT *et al.*, 1949, p. 148). Unfortunately, this has not been possible with lobster nerve homogenates. In lobster nerve the fibres are very loosely bound together and tend to separate and slip through the homogenizer undamaged, so that it is not possible to achieve complete rupture of the cells without excessively long homogenization. Moreover, the mitochondria show a wide range of sizes from 0·2 to 1 μ, which partly includes that generally assigned to the submicroscopic particles or microsomes; hence, any mitochondrial preparation made from this material will be heavily contaminated with

TABLE 1.—DISTRIBUTION OF CYTOCHROME OXIDASE AND SUCCINIC DEHYDROGENASE IN LOBSTER NERVE

Fraction	Cytochrome oxidase		Succinic dehydrogenase	
	Specific activity	% Total activity	Specific activity	% Total activity
Homogenate	1·4	100	0·052	100
Nuclear	1·8	22	—	—
Mitochondria	11·9	42	0·837	28
Supernatant	1·0	25	0·057	45
Recovery, per cent		89		73

other particles. Indeed, electron micrographs of such preparations reveal a variety of material, only some of which is morphologically similar to the mitochondria seen in sections of intact nerve fibres. This problem can be shown in another way. If one accepts the current belief (SCHNEIDER and HOGEBOOM, 1951) that cytochrome oxidase is associated exclusively with the mitochondria, then the presence of cytochrome-oxidase activity can be taken as an indication of the presence of mitochondrial material. It was found that even prolonged centrifugation at 45,000 g, which should sediment submicroscopic particles as well as mitochondria, would not remove all the cytochrome oxidase activity. Consequently the centrifugal speed yielding the preparation of highest specific activity (per milligram protein) was chosen, sacrificing yield for the sake of purity. This must be borne in mind when interpreting the data.

Table 1 shows that succinic dehydrogenase and cytochrome oxidase activities were concentrated nine- and sixteen-fold respectively upon isolation of the "mitochondrial" fraction. This indicates that these enzymes are concentrated in the mitochondria, although the yield was only 42 and 28 % of the total activity. Cytochrome oxidase was also present in considerable excess over succinic dehydrogenase, a finding in keeping with other tissues (SCHNEIDER and POTTER, 1943). The same degree of purification was achieved using squid giant fibre (Table 2), although the actual level of activity in the whole fibre and axoplasm was considerably greater. This most likely indicates that the preparations are purer when obtained from squid fibre, since there is considerably less contaminating connective tissue protein. As shown in Table 2, the activity of the intact sheath, however, was quite low (8 %) compared with the axoplasm of the whole fibre. This point will be discussed later. The difference

between intact sheath and its homogenate is probably one of permeability to the substrate. We were able to detect only very low succinic dehydrogenase activity in squid nerve, although COOPERSTEIN and LAZAROW (1950) found significant amounts.

TABLE 2—DISTRIBUTION OF CYTOCHROME OXIDASE IN SQUID GIANT NERVE FIBRE

Fraction	Number of experiments	Specific activity
Whole nerve	2	14·7
Axoplasm	3	10·2
Mitochondria	2	83·4
Isolated sheath		
Intact	3	1·2
Homogenate	4	2·3
Mitochondria	1	18·0

Specific activity = $-\Delta$ log [ferricytochrome *c*]/mg protein/min.

The lobster nerve mitochondrical preparations were capable of carrying out oxidative phosphorylation in a system containing glucose, hexokinase, and potassium fluoride. The addition of bovine-serum albumin to the incubation medium, suggested

TABLE 3—OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA FROM LOBSTER PERIPHERAL NERVE

Substrate	μA O_2 utilized per mg protein per hour	μM P esterified per mg protein per hour	P/O
Succinate	0·316	0·246	0·78
	0·083	0·051	0·61
Fumarate	0·541	0·510	0·95
	0·120	0·092	0·77
	0·080	0·124	1·6
Malate	0·231	0·260	1·1
	0·051	0·069	1·4
Citrate	0·398	0·274	0·69
	0·068	0·069	1·0
α-ketoglutarate	0·044	0·068	1·5
	0·054	0·073	1·4

Each flask contained the following: 5 μM substrate, 0·005 μM cytochrome *c*, 0·6 mg bovine serum albumin, 1 μM KF, 5 μM $MgCl_2$, 2 μM Na_2-ATP, 3 μM glucose, 2 mg yeast hexokinase, 6 μM Na_2HPO_4, in 0·04 M glycylglycine buffer, adjusted to pH 7·5 with KOH. Mitochondria from about 8 g nerve. Total volume 0·6 ml. Hexokinase and $MgCl_2$ tipped in at zero time. $T = 20$ C. Incubation time 2 hours.

by SACKTOR (1954), improved the results and tended to keep the mitochondria from clumping. The results from experiment to experiment varied over a wide range. However, variations in oxygen consumption usually had a concomitant variation in phosphate utilization, so that the P/O ratio did not show quite so much variability. From the data in Table 3 it can be seen that P/O ratios in excess of 1·0 were obtainable

in some cases. It is possible that better results might have been obtained if the measurements had been made after an extended period of equilibration. The oxygen uptake tended to be quite erratic for 45 min after the side arm contents were tipped in. After this it became quite uniform for as long as four hours. It might have been preferable to take the zero value for phosphate at this point instead of at zero time.

The respiration experiments on squid giant fibre and sheath were interrupted prematurely by the hurricanes and floods which ended the squid season, so that only a small number of experiments could be completed. However, because the data that *were* obtained were consistent, it was considered desirable to include the results in preliminary fashion in this report. That the results may have been fairly typical was indicated by the fact that recalculation of the data on the basis of two rough measurements of the wet-weight/protein ratio gave an oxygen consumption per gram wet weight for the intact fibre comparable with that obtained by CONNELEY (1952). The important observation in these preliminary experiments was that the isolated sheath accounted for approximately 20% of the total respiration of the nerve fibre, based on the assumption that 55% of the protein in the fibre is in the sheath (range of measurements 46–67%). A more extended series of measurements will be undertaken when squid are again available.

VOL
1
1956/

DISCUSSION

Particulates isolated from lobster leg nerves by standard differential centrifugation techniques are morphologically similar to those seen clustered at the Schwann cell-axon interface by GEREN and SCHMITT (1954). Concentrated in the particulates are respiratory enzymes, such as succinic dehydrogenase and cytochrome oxidase, although the evidence does not confine these enzymes exclusively to this cell fraction. These particles are capable of carrying out oxidative phosphorylation, behaving in this respect like typical mitochondria. Hence, presumably, they can serve as a source of energy for the functions of the nerve fibre. Such an interpretation would be consistent with the concentration of the mitochondria at the axon-Schwann cell interface (GEREN and SCHMITT, 1954), where the coupling of energy and impulse propagation is thought to occur (SCHMITT and GEREN, 1956).

When nerve mitochondria are isolated in a relatively uncontaminated state, as when squid axoplasm is used as the source, the specific activities (83·4, see Table 2) compare favourably with those measured by COOPERSTEIN *et al.* (1951), on a variety of mammalian tissues. Since the enzymatic activity of these organelles is of a high order, perhaps the low metabolic activity characteristic of peripheral nerve as a whole tissue is more a reflection of the basis used for expressing the results: the region responsible for the activity may represent but a small fraction of the fibre weight and may be highly localized (i.e., primarily in the Schwann cell).

The question of the basis for expressing experimental results assumes great importance when one considers the measurements on isolated sheath preparations. According to Table 2, the metabolic activity of the sheath appears to be a good deal lower than that of the axoplasm. However, electron micrographs of the isolated sheath show that, even when carefully cleaned, the preparation is still predominantly connective tissue, with the Schwann cells as a thin layer upon the inner surface. It has been possible to estimate the amount of connective tissue in the sheath, making use of the limited solubility of collagen and elastin in 0·1-N NaOH (LOWRY *et al.*, 1941). The values

ranged between 65 and 80% of the total protein, and are likely to be on the low side, so that at most 25% of the sheath protein is in the cellular component.* If the cytochrome oxidase activity of a sheath homogenate (2·3, Table 2) is appropriately corrected for connective-tissue content, a value of 9·2 is obtained, which is close to that for the axoplasm itself. In other words, when results are expressed *per unit of metabolically active protein*, the Schwann cell may be considered as being at least as active as the axon, and perhaps considerably more so. The observations presented here, as well as others (NACHMANSHON, 1952; LIBET, 1948) demonstrating concentration of certain enzymes in the sheath, indicate that the Schwann cells are very active metabolically, a result consistent with the hypothesis that the Schwann cell may play a significant role in the chemical metabolism of peripheral nerve.

SUMMARY

Mitochondria from lobster leg and claw nerves have cytochrome oxidase and succinic dehydrogenase concentrated in them, and can couple oxidation and phosphorylation, behaving thus like typical mitochondria. Cytochrome oxidase is also concentrated in squid giant fibre mitochondria. Preliminary respiratory data on isolated squid fibre sheaths indicate that the sheath accounts for approximately 25% of the respiration of the intact fibre. Correction of the sheath data for connective tissue content (*ca.* 75%) gives values as great as those for axoplasm. It is suggested that the Schwann cell may play a significant role in the chemical metabolism of peripheral nerve.

VOL. 1 56/57

Acknowledgements—The author is indebted to Professor FRANCIS O. SCHMITT, who suggested this investigation as part of a broad programme of nerve research in this laboratory. Appreciation is expressed also to Dr. B. GOLDBERG, who performed the squid-nerve respiration experiments, to Dr. BETTY B. GEREN for the electron micrographs, and to Miss JOAN A. LENTINO for technical assistance.

REFERENCES

ABOOD L. G. and GERARD R. W. (1953) *Fed. Proc.* **12,** 3.
CONNELLY C. M. (1952) *Biol. Bull.* **103,** 315.
COOPERSTEIN S. J. and LAZAROW A. (1950) *Biol. Bull.* **99,** 356.
COOPERSTEIN S. J., LAZAROW A., and KURFESS N. J. (1950). *J. Biol. Chem.*, **186,** 129.
COOPERSTEIN S. J. and LAZAROW A. (1951) *J. Biol. Chem.* **189,** 665.
GEREN B. B. and SCHMITT F. O. (1954) *Proc. Nat. Acad. Sci.* **40,** 863.
KOECHLIN B. A. (1955) *J. Biophys. Biochem. Cytol.* **1,** 511.
LEWIS P. R. (1952) *Biochem. J.* **52,** 330.
LIBET B. (1948) *Fed. Proc.* **7,** 72.
LOWRY O. H., GILLIGAN D. R., and KATERSKY E. M. (1941) *J. Biol. Chem.* **139,** 795.
LOWRY O. H. and LOPEZ J. A. (1946) *J. Biol. Chem.* **162,** 421.
LOWRY O. H., ROSENBROUGH N. J., FARR A. L., and RANDALL R. J. (1951) *J. Biol. Chem.* **193,** 265.
MAXFIELD M. (1953) *J. Gen. Physiol.*, **37,** 201.
MULLINS L. J. (1953) *Amer. J. Physiol.* **175,** 358.
NACHMANSOHN D. (1952) In MCELROY W. D. and GLASS, B. *Phosphorus Metabolism* (Johns Hopkins Press, Baltimore).

* Electron micrographs of thin sections of squid giant fibres, made in other studies by Dr. B. B. GEREN, show that, depending upon how well the sheaths were cleaned, a few very thin (0·1 to 0·5 μ) fibrocytes were included in the sheath. Nothing is known about the chemical metabolism of these cells and, since it was difficult even to guess at their volume relative to the Schwann cells, the figures as given refer merely to the "cellular component".

SACKTOR B. (1954) *J. Gen. Physiol.* **37,** 343.
SCHMITT F. O., BEAR R. S., and SILBER R. H. (1939) *J. Cell. Comp. Physiol.* **4,** 351.
SCHMITT F. O. and GEREN B. B. (1956) *Protoplasma* (in press).
SCHNEIDER W. C. and HOGEBOOM G. H. (1951) *Cancer Res.* **11,** 1.
SCHNEIDER W. C. and POTTER V. R. (1943) *J. Biol. Chem.* **149,** 217.
SCHOLANDER P. F., CLAFF C. L., ANDREWS J. R., and WALLACH D. F. (1952) *J. Gen. Physiol.* **35,** 375.
SIEKEVITZ P. and POTTER V. R. (1953) *J. Biol. Chem.* **200,** 187.
SILBER R. H. (1941) *J. Cell. Comp. Physiol.* **18,** 21.
SILBER R. H. and SCHMITT F. O. (1940) *J. Cell. Comp. Physiol.* **16,** 247.
UMBREIT W. E., BURRIS R. H., and STAUFFER J. F. (1949) *Manometric Techniques and tissue Metabolism* (Burgess, Minneapolis).

VOL
1
1956/

NEWS AND NOTES

THE METABOLISM OF THE NERVOUS SYSTEM

THE proceedings of the Second International Neurochemical Symposium, to be held at Aarhus, Denmark, 1956, will be published by Pergamon Press Ltd. They will be edited by DEREK RICHTER, Director of the Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff.

The volume will contain fifty papers and reviews on the biochemistry and on chemical aspects of the anatomy and physiology of the nervous system. The contributions include:

Electron microscopy of the nervous system	H. FERNANDEZ-MORAN (Caracas)
Structure of the nerve membrane	F. O. SCHMITT (Boston)
Electrolytes and nerve activity	R. D. KEYNES (Cambridge)
Cytochemistry	A. POPE (Boston)
General metabolism of tissue preparations	J. H. QUASTEL (Montreal)
General metabolism of the brain	S. S. KETY (Washington)
Metabolism of tissue particles	G. H. HOGEBOOM (Washington)
Cytological aspects of nucleic acid metabolism	L. EINARSON (Aarhus)
Carbohydrate metabolism	H. A. KREBS (Oxford)
Metabolism of coenzymes	F. LIPMANN (Boston)
Lipid metabolism	R. J. ROSSITER (London, Ontario)
Fatty acid metabolism	F. LYNEN (Munich)
Metabolism of proteins and amino acids	H. WAELSCH (New York)
Mechanism of methylation	G. L. CANTONI (Washington)
Acetylcholine	W. FELDBERG (London)
Noradrenalin and adrenalin in the central nervous system	M. VOGT (Edinburgh)
Biochemistry and metabolism of serotonin	S. UDENFRIEND (Washington)

2nd EUROPEAN CONFERENCE ON PSYCHOSOMATIC RESEARCH

THE conference was held in Amsterdam from 17 to 21 April 1956. The programme included the following subjects:

1. The philosophical and psychiatric basis of psychosomatic medicine.
2. Psychosomatic aspects of arterial hypertension.
3. Survey of activities of the psychosomatic research unit at the Wilhelmina-Gasthuis.
4. Animal behaviour and psychosomatic medicine.
5. Body build, character, and disease.

SYMPOSIUM ON HYPOTENSIVE DRUGS AND THE CONTROL OF VASCULAR TONE IN HYPERTENSION

THE symposium took place in the auditorium of the Wellcome Building, London, on 5 and 6 April. It was organized by the British Pharmacological Society, the Physiological Society, the Royal Society of Medicine, the Fine Chemicals Group, the Biochemical Society, the Pharmaceutical Society, the Association of Anaesthetists, and the Society of Endocrinology.

The following papers were presented:

Opening remarks by Sir CHARLES HARINGTON, F.R.S.

CHEMICAL and BIOCHEMICAL ASPECTS

Chairman: Professor F. BERGEL

H. R. ING (Oxford): Structure-action relationship of hypotensive drugs

H. BLASCHKO (Oxford): Biochemical principles in relation to hypotensive drug action

THE PHARMACOLOGY OF HYPOTENSIVE DRUGS

Chairman: Professor J. H. BURN, F.R.S.

J. TRIPOD (Basle): Some pharmacological differences between hypotensive drugs, with special reference to hydralalazine and reserpine

MARTHE VOGT (Edinburgh): Theories concerning the site and mode of action of reserpine

W. S. PEART (London): Substances antagonistic to adrenaline and noradrenaline

J. G. WIDDICOMBE (London): Veratrum alkaloids

ELEANOR ZAIMIS (London): Ganglionic blocking substances

CLINICAL APPLICATIONS OF HYPOTENSIVE DRUGS

Chairman: Professor J. MCMICHAEL

J. MCMICHAEL (London): Results of methonium treatment in malignant hypertension; five years' follow-up

F. SMIRK (Dunedin): Principles and details in hypotensive therapy

G. A. PERERA (New York): Hypotensive drugs: clinical evaluation under controlled conditions

THE CONTROL OF VASCULAR TONE IN HYPERTENSION

Chairman: Professor CLIFFORD WILSON

CLIFFORD WILSON (London): Clinical and experimental indications

B. FOLKOW (Gothenburg): Structural, myogenic, humoral, and nervous factors controlling peripheral resistance

M. A. FLOYER and J. M. LEDINGHAM (London): Renal and extrarenal factors in experimental hypertension

The proceedings of this symposium, including discussions, will be published shortly by Pergamon Press Ltd.

VOL 1 1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 93–100, Pergamon Press Ltd., London.

CYTIDINE AND URIDINE REQUIREMENT OF THE BRAIN*†

A. GEIGER and S. YAMASAKI
Department of Psychiatry, Neuropsychiatric Institute,
University of Illinois College of Medicine, Chicago

(*Received* 12 *March* 1956)

THE nutritional requirements of the brain were investigated previously in perfusion experiments on cats. It has been shown that the carbohydrate metabolism of the brain, and its survival, is dependent on substances which are produced or released by the liver into the blood circulation. In the absence of the liver, aerobic glycolysis developed in the brain, followed later by impermeability to glucose, reducing the survival time of the perfused brain to about one hour. Insertion of the liver into the circulation corrected the faulty carbohydrate metabolism of the brain and prolonged its survival by several hours. (GEIGER, MAGNES, TAYLOR, and VERALLI, 1954).

It was found in the present experiments that, by adding small amounts of cytidine and uridine to the perfusion fluid, it was possible to maintain the perfused brain in good functional condition with a normal carbohydrate metabolism for four hours or longer without the liver. These experiments indicate the necessity of these nucleosides for the maintenance of normal brain functions and metabolism. An attempt is also made to elucidate the mechanism of action of these substances on brain metabolism.

EXPERIMENTAL METHODS

The method of GEIGER and MAGNES (1947) was used for the isolation of brain circulation and for the perfusion, with some modifications. In spite of careful occlusion of all the major venous outlets of the head other than those from the brain, some blood, amounting to about 1% of the total circulation, was seeping through the muscles of the face into the brain circulation. In order to avoid this error, several tight ligatures were put across the bunched facial muscles. The muscles from the skull were peeled off down to the zygomatic arc including the ears, and a hard knot was pressed into the palatum with a wire ligature. The clips by which the vertebral sinuses are compressed have been modified so as to make it possible to insert them through the dorsal foramen magnum. The perfusion apparatus has been somewhat modified. The arterio-venous oxygen differences were recorded by a modified oxymeter, cathode-ray follower circuit, and Speedomax. The rotating blood oxygenator was supplanted by a static vertical tube filled with marbles through which the blood and oxygen ran in counter-current. Two photoelectric cells were substituted for the electrolytic chamber in the blood-flow recorder.

The "simplified blood" used for the perfusion was prepared in the following way: defibrinated and filtered ox-blood was washed in the centrifuge with a Krebs-Ringer solution and suspended in about 10 volumes of Krebs-Ringer solution for one to two hours, centrifuged and suspended once again. After two hours, the erythrocytes were centrifuged again and placed into half-inch

* Supported by the U.S. Public Health Service, Division of Neurological Diseases and Blindness.
† Preliminary report presented at the meeting of the Federation of American Societies for Experimental Biology, April 1956.

cellophane sausage casings, which were tied and suspended in a large container of Krebs-Ringer solution in the cold room. With frequent changes of the Ringer solution, the erythrocytes were dialysed for at least two days before use. To the Ringer solution, as well as to the erythrocytes, penicillin and streptomycin were added. The perfusion blood was made up of 30% of the dialysed erythrocytes suspended in Krebs-Ringer solution containing 7% native bovine serum albumin (Pentex). The albumin had been passed through cation and anion exchange columns before use. In some cases it was also dialysed. The "simplified blood" was adjusted to pH 7·3 with isotonic sodium bicarbonate solution.

Glucose was determined with NELSON's method (1944) and by measuring reduction before and after treatment of the filtrates with glucose oxidase. Lactic acid was determined by LE PAGE's method (1944). For the determination of the lipid and nucleic acid fractions, brain samples weighing 0·2 g to 0·3 g were homogenized in cold 0·5 N perchloric acid (PCA) and fractionated according to SCHNEIDER's procedure (1945). The lipids were extracted with ethanol-ether (3 : 1), nucleic acid derivatives with hot 5% perchloric acid. For the determination of lipid-galactose, the method of BRAND and SPERRY (1941) was used. Total nitrogen has been determined on all fractions after combustion. For determining the purine bases and pyrimidine nucleosides, the hot PCA extracts were hydrolysed for one hour in 5% perchloric acid, neutralized with KOH, cooled for three hours in ice to remove the precipitated $KClO_4$, and the supernatant passed through a short Dowex-50 column in 2 N HCl. The column was eluted with 2 N HCl to which 3 N HCl was gradually added. The absorption spectra of the effluent and of each eluate was measured in the Beckman spectrophotometer, and their nitrogen content was determined after digestion. After evaporation to dryness, each fraction was taken up in 0·1 ml of water, spotted on Whatman filter paper, and chromatographed in a butanol-acetic acid-water or butanol-formic acid-water system.

RESULTS

The course of perfusion experiments without and with the liver in the circulation has been shown previously (GEIGER *et al.*, 1954). These experiments demonstrated aerobic lactic acid production, development of impermeability to glucose, and short survival-time of the preparation in the absence of the liver, as well as the maintenance of normal carbohydrate metabolism and survival of the brain for two to three hours when an isolated liver was inserted into the circulation.

A representative experiment in Fig. 1 shows the effects of uridine and cytidine, when these compounds were added to the perfusion blood after the brain has been perfused with the simplified blood for about one hour. The electrocorticogram in Fig. 2 shows that the electrical activity of the brain was practically extinguished after one hour perfusion with the simplified blood, and has been restored by the addition of uridine and cytidine. Also, the corneal and pupillary reflexes which could not be elicited before the addition of these substances returned to normal in the presence of cytidine and uridine. Furthermore, if these substances were repeatedly added in 10 mg quantities to the perfusion blood (400 ml), the brain maintained good physiological activity, with a normal EEG, even after four hours of perfusion. At this time, vigorous convulsions could be elicited with metrazol (Figs. 1 and 2).

Cerebral oxygen consumption and blood-flow were not significantly influenced by the addition of cytidine or uridine or both, except that the oxygen consumption and blood-flow remain steady and constant during a perfusion lasting four hours or longer in the presence of these two substances in the perfusion blood (Figs. 1 and 2).

The carbohydrate metabolism of the brain, however, was considerably improved after the addition of cytidine to the perfusion blood. Usually, after 50 to 75 min. of perfusion with the simplified blood, the lactic acid concentration in the brain increased up to 300–400 mg per cent and at the same time the glucose concentration decreased to between 10–40 mg per cent. If, at this point, first uridine and then

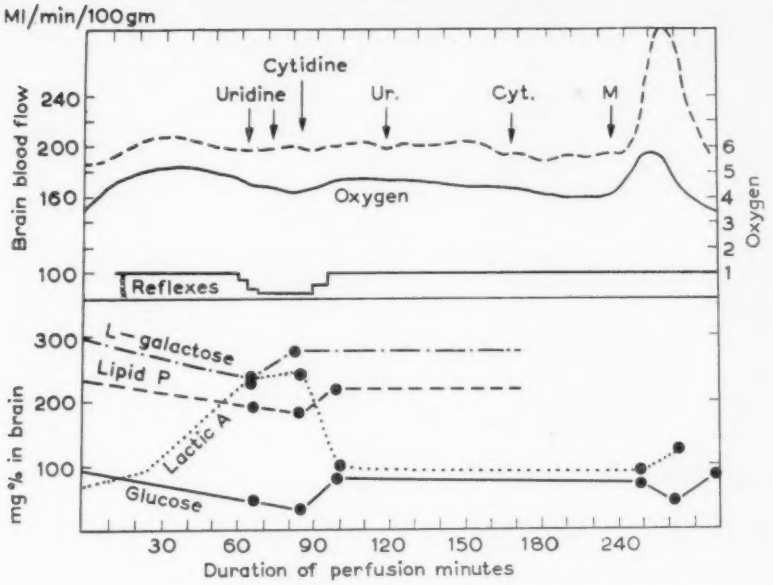


FIG. 1. The upper graph shows the course of perfusion during a 4½ hour period. The arrows show when uridine, cytidine (10 mg of each), and metrazol (M) were added to the perfusion blood. The lower graph shows the analyses on biopsy samples taken as indicated during the perfusion.

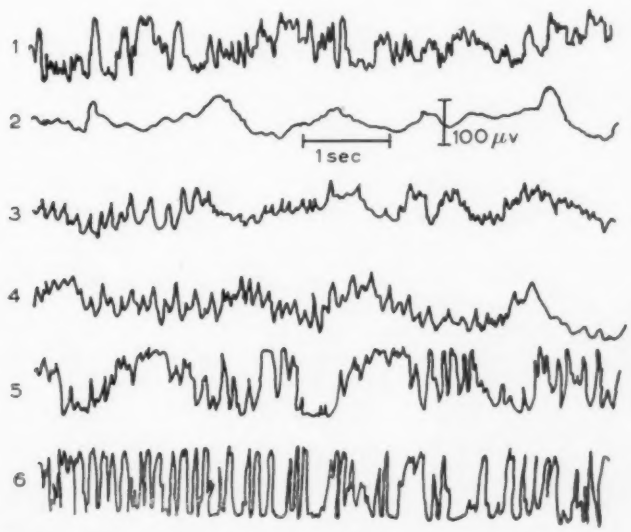


FIG. 2. The EEG records taken during a perfusion experiment of over 4 hours duration. Electrodes on frontal cortex.

1. 10 minutes after start of perfusion.
2. 60 minutes after start of perfusion.
3. 7 minutes after addition of uridine (68 min perfusion).
4. 10 minutes after addition of cytidine (88 min perfusion).
5. After 4 hours of perfusion, and repeated additions of uridine and cytidine.
6. Metrazol convulsions after 4½-hours perfusion.

cytidine were added, then after the addition of 10–20 mg of cytidine, the lactic acid concentration in the brain decreased very rapidly to 80–100 mg per cent and the glucose concentration increased to 60–70 mg per cent (with 100 mg per cent glucose in the blood). Moreover, if the addition of these nucleosides was repeated from time to time, the carbohydrate metabolism of the brain remained normal for the rest of the perfusion, for over four hours.

The analytical results on brain cortex samples, taken during perfusion, and presented in Tables 1 and 2, are typical of twenty-one experiments (of twenty-two) made so far with the addition of cytidine and uridine. Experiments without additions (Type *A*) and with an isolated liver inserted into a by-pass of the brain circulation (Type *B*) are also included in the tables for comparison, although experiments of this type (of which hundreds were made during the last few years) have been reported previously.

Table 1 contains glucose and lactic acid analyses on biopsy samples of the brain taken during perfusion at times stated, and shows the effect of cytidine and uridine.

The results of these experiments show that the addition of cytidine and uridine prevents the accumulation of lactic acid and the depletion of glucose in the brain Table 1). Even when lactic acid accumulated during the first 50 to 60 min of perfusion, the lactic acid concentration significantly diminished after these pyrimidine nucleosides had been added to the perfusion blood. If cytidine and uridine were present in the perfusion blood throughout the perfusion, the lactic acid and glucose concentrations in the brain remained normal for three to five hours of perfusion.

In a number of experiments, perfusion was continued for 30 to 60 min beyond the stage of maximum accumulation of lactic acid in the brain (300 to 400 mg %) without adding cytidine and uridine. In these cases, the addition of cytidine and uridine at this late stage was ineffective. Thus, the addition of cytidine and of uridine was as effective in maintaining normal brain functions and carbohydrate metabolism, as was the inclusion of a liver in the perfusion system.

The galactoside content of the brain was examined on biopsy samples taken during the course of the perfusion, before and after the addition of uridine, in view of the known activity of uridine diphosphate glucose in the glucose-galactose conversion (LELOIR, 1951). The results shown in Fig. 1 and Table 2 suggest that when the galactoside content of the brain is reduced by perfusion, the addition of uridine tends to restore it to its original level.

It has recently been shown by KENNEDY and WEISS (1955) that cytidine-diphosphate-choline catalyses lecithin formation, and cytidine-diphosphate-ethanolamine the synthesis of cephalins (KENNEDY, 1956). In view of these findings, lipid-phosphorus was determined in the brain cortex during the course of the perfusion. The results shown in Fig. 1 and Table 2 indicate a loss of phosphatides during the perfusion preceding the addition of cytidine, and their resynthesis after cytidine was been added to the perfusion blood.

Table 2 shows that lipid galactose and lipid P decreased during perfusion with the simplified blood. The addition of cytidine and uridine to the perfusion blood prevents the depletion of these lipids, or if added at a later stage of perfusion, tends to restore the cerebroside and phospholipid concentrations in the brain. This restoring effect of cytidine and of uridine on the brain lipid content was best seen in experiments where the galactolipid concentration was low at the start of the perfusion.

VOL
1
1956/

TABLE 1.—EFFECT OF URIDINE AND CYTIDINE ON GLUCOSE AND LACTATE IN THE BRAIN

Type of experiment	Perfusion time	Additions	mg in 100 g brain*	
			Glucose	Lactic acid
A	0	None		
	30		78	121
	50		41	293
	65		36	331
	90		24	387
B	0	With liver in circulation		
	30		84	102
	60		77	106
	90		73	98
		Exchanged liver		
	150		69	115
	180		63	120
C	0	15 mg each of cytidine and uridine		
	30		73	91
	60		71	88
	65	15 mg each of cytidine and uridine		
	110		70	93
	115	15 mg each of cytidine and uridine		
	155			
	185		68	106
	188	10 mg each of cytidine and uridine		
	240		61	110
D	0	None		
	60		36	326
	63	15 mg of uridine		
	78		30	351
	80	15 mg of cytidine		
	95		67	126
	98	20 mg of uridine and cytidine		
	150		61	114

* The concentration of glucose in the blood was between 100 and 120 mg per 100 ml.

It should be pointed out that in the present experiments, the preparation of the blood was different from that in previous ones (GEIGER *et al.*, 1954). While previously, bovine erythrocytes were suspended in a large amount of Ringer and centrifuged four or five times before use, in the present experiments the erythrocytes were suspended only twice and dialysed afterwards. Thus, the possibility exists that, in the present blood preparation, platelets, and possibly white blood cells were retained. The possible effect of these components is being investigated.

There are, also, definite changes in the nucleic acid composition of the brain

cortex during perfusion, Before uridine and cytidine are added there is a small increase of pyrimidine bases and a larger decrease of purine bases in the brain. These changes are partly reversed by the addition of uridine and cytidine.

TABLE 2.—EFFECT OF URIDINE AND OF CYTIDINE ON THE LIPID GALACTOSE AND LIPID P CONTENT OF BRAIN

Type of experiment	Perfusion time	Additions	mg in 100 g brain	
			lipid galactose	lipid-P
A	0	None		
	30		326	186
	60		282	131
	90		211	131
C	0	15 mg each of cytidine and uridine		
	30		339	194
	60		319	187
	65	15 mg each of cytidine and uridine		
	110		330	196
	115	15 mg each of cytidine and uridine		
	155		343	202
D	0	None		
	60		221	153
	65	15 mg of uridine		
	78		266	147
	80	15 mg of cytidine		
	95		280	191
	98	20 mg each of uridine and cytidine		
	150		288	195

It was observed in previous perfusion experiments that the addition of purine nucleotides and nucleosides, particularly of adenosine triphosphate, caused a sudden large reduction of the cerebral blood flow and of oxidation, the disappearance of reflexes and of the electrocorticogram. Adenosine triphosphate was most active. Two to three milligrams of this substance in 100 ml of blood extinguished brain activity for 5 to 10 min, until it was removed from the brain. This depressing effect of ATP could seldom be reversed by subsequent perfusion with ATP-free blood, especially if larger amounts of ATP were added (10–20 mg%), since the greatly increased cerebral vascular resistance prevented the washing out of these substances from the brain. In the present experiments, after the addition of cytidine and uridine to the ATP-treated brain, reflexes and EEG returned, and the blood-flow became normal within 2 to 5 min. If ATP was added to the perfusion blood when this already contained cytidine and uridine, the ATP effect was only noticeable when ten times more ATP was added than before. These experiments show that these

VOL 1 1956/

pyrimidine nucleosides antagonize the depressing effect of ATP on brain oxidation and cerebral blood flow.

DISCUSSION

The preceding experiments show that the presence of cytidine and uridine in the blood is essential for maintaining normal brain functions, and that in perfusion experiments the effect of their presence is at least equivalent to that of the liver. It is not known how much of these substance circulate in the blood, or whether these substances are put out by the liver into the circulation.

The loss of galactosides and of phospholipids from the brain during perfusion is not surprising in view of previous findings of GEIGER, MAGNES and GEIGER (1952), which show that during glucose-free perfusion the brain maintains a high rate of oxidation with an R.Q. (Respiratory Quotient) reduced to 0·6–0·8, and of observations of ABOOD and GEIGER (1955) which show the breakdown of phospholipids under such conditions. As mentioned above, during perfusion without the liver in the circulation, the oxidation of carbohydrates in the brain is progressively impaired and carbohydrate metabolism is restricted to aerobic glycolysis. Nevertheless, the brain continues to take up oxygen. This oxygen uptake may well be due to oxidation of lipids. Furthermore, it has been shown by GEIGER, YAMASAKI, and LYONS (1956) that during stimulation of the brain, rapid changes occur in the lipid composition of the brain cortex. Rapid turnover of particular lipid fractions in the brain was shown by DAWSON and RICHTER (1950) and STREICHER and GERARD (1954), and by HOKIN and HOKIN (1954). The effect of added uridine and of cytidine on brain lipid content can thus be interpreted in the light of the role of uridine-diphosphate glucose in the glucose transformation (LELOIR, 1951), and of cytidine diphosphate-choline or -ethanolamine in lipid synthesis (KENNEDY and WEISS, 1955).

The main effect of cytidine and uridine on the physiological activity of the brain lies probably in preventing the accumulation of lactic acid in the brain. During perfusion, in the absence of the liver or of these nucleosides, lactic acid in the brain reached very high concentrations (300–400 mg per cent) and is probably responsible for the cessation of all physiological activity. The mechanism by which cytidine and uridine restore carbohydrate oxidation in the brain and eliminate aerobic glycolysis is unknown.

SUMMARY

(1) It has been previously shown that the carbohydrate metabolism of the brain and its survival is dependent on substances produced or released by the liver into the blood circulation. In the absence of the liver, aerobic glycolysis, followed by impermeability to glucose developed in the brain, reduces the survival time of the perfused brain to about one hour (BRAND and SPERRY, 1941; DAWSON and RICHTER, 1950). Insertion of the liver into the circulation or addition of fresh liver extract corrected the faulty carbohydrate metabolism of the brain and prolonged its survival by several hours.

(2) In the present experiments, small amounts of cytidine and uridine added to the perfusion fluid maintained the perfused brain in good functional condition with a normal carbohydrate metabolism for four hours or longer without the liver.

(3) After the brain had been perfused for one hour, the addition of cytidine and uridine restored the carbohydrate metabolism of the brain to normal, reducing the

lactic acid concentration and increasing the glucose concentration. The concentration of galacto-lipids and phospholipids which were reduced during the previous perfusion were also restored to their initial level.

(4) Changes in nucleic acid components were observed during perfusion. These changes were partially reversed by cytidine and uridine. It is suggested that the presence of cytidine and of uridine are necessary for the maintenance of normal brain functions.

REFERENCES

BRAND F. C. and SPERRY W. M. (1941) *J. Biol. Chem.* **141,** 545.
DAWSON R. M. C. and RICHTER D. (1950) *Proc. Roy. Soc.* **B137,** 253.
GEIGER A. and MAGNES J. (1947) *Amer. J. Physiol.* **149,** 517.
GEIGER A., MAGNES J., TAYLOR R. M., and VERALLI M. (1954) *Amer. J. Physiol.* **177,** 138.
GEIGER A., YAMASAKI S., and LYONS R. *Amer. J. Physiol.* (In press).
HOKIN M. D. and HOKIN L. E. (1954) *J. Biol. Chem.* **209,** 549.
KENNEDY E. Personal Communication.
KENNEDY P. K. and WEISS S. B. (1955) *J. Amer. Chem. Soc.* **77,** 250.
LELOIR L. (1951) *Arch. Biochem. and Biophys.* **33,** 186.
LEPAGE G. A. (1946) Manometric techniques and related techniques for the study of tissue metabolism. UMBREIT W. W., BURRIS, F. H., and STAUFFER J. F. Burgess Publishing Co., Minneapolis.
NELSON N. (1944) *J. Biol. Chem.* **153,** 375.
SCHNEIDER W. C. (1945) *J. Biol. Chem.* **161,** 293.
STREICHER E. and GERARD R. W. (1954) *Proc. Soc. Exp. Biol., N.Y.* **85,** 174.

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 101–108, Pergamon Press Ltd., London.

THE ISOLATION FROM BRAIN TISSUE OF A TRYPSIN-RESISTANT PROTEIN FRACTION CONTAINING COMBINED INOSITOL, AND ITS RELATION TO NEUROKERATIN*

F. N. LEBARON† and JORDI FOLCH
The McLean Hospital Research Laboratory, Waverley, Mass., and
the Department of Biological Chemistry, Harvard Medical School, Boston, Mass.

(*Received* 30 *May* 1956)

INTRODUCTION

IT has been well established that *meso*-inositol exists in brain in both free and combined states. More than fifty years ago, THUDICHUM (1901) reported the presence of free inositol, and this observation has since been amply confirmed. More recent work (FOLCH and WOOLLEY, 1942) demonstrated the occurrence of inositol combined in brain lipides, and resulted in the isolation from brain of diphosphoinositide (FOLCH, 1949), a phosphatide which contains inositol metadiphosphate as a constituent. Results obtained in this laboratory in the course of work on the nature of neurokeratin have established the presence in brain tissue of a second form of combined inositol, namely, that of a phosphoinositide combined to protein. This paper reports these findings and some other observations bearing on the nature of neurokeratin. Preliminary reports of this work have already appeared (FOLCH and LEBARON, 1951, 1953).

Neurokeratin is a fraction of brain proteins first isolated by EWALD and KÜHNE (1877) which can be summarily described as a proteolytic enzyme-resistant protein residue obtained primarily from nerve tissue myelin, and which has traditionally been considered to be associated with the protein network of the myelin sheath. The classical procedure for preparing neurokeratin consists essentially of two major operations; namely, the quantitative removal of lipides with organic solvents, and the subsequent treatment of the proteins thus obtained, with digestive enzymes. The procedure takes several months, and involves such drastic steps as continuous extraction with hot ethanol for four weeks, digestion with pepsin in dilute hydrochloric acid for three weeks, and, in some of its versions, treatment with hot alkali. The product is a protein residue insoluble in water and in dilute acids and bases, rich in sulphur, and resistant to the action of proteolytic enzymes. The preparations obtained by various workers differed somewhat in composition, and BLOCK (1951) concluded that neurokeratin is most likely an artifact, derived from a definite group of substances specific to nervous tissue.

The work which led to the isolation of the protein fraction containing combined inositol was an attempt to develop a method of preparation of neurokeratin less

* This investigation was supported in part by Grant No. RG–1176 from the National Institutes of Health, U.S. Public Health Service. Part of the data in this paper is taken from a thesis submitted by F. N. LEBARON to the Division of Medical Sciences of Harvard University in partial fulfilment of requirements for the degree of Doctor of Philosophy.

† U.S. Public Health Service Postdoctorate Research Fellow of the National Institute of Mental Health 1951–1952.

drastic than the classical procedure. The method which was eventually developed consisted of extracting brain tissue with chloroform : methanol, which presumably removed all the tissue lipides (FOLCH *et al.*, 1951), washing the insoluble tissue residue with water, and finally treating it with trypsin. The resulting trypsin-resistant protein residue (TRPR) was similar to neurokeratin in that it was insoluble in water and in dilute acid or base, that it was rich in sulphur, and that it was resistant to the action of pepsin, trypsin, papain, and erepsin. However, in sharp contrast to neurokeratin, which is essentially free of phosphorus (and, as was found later, of combined inositol), our material contained fairly large concentrations of both phosphorus and combined inositol.

This phosphorus could not be acid-soluble phosphorus or phosphatide-phosphorus in the accepted sense, because these would presumably have been removed during the preparative procedures. By using the partition methods of SCHNEIDER (1945) and SCHMIDT and THANNHAUSER (1945), it was found that this was not nucleic acid phosphorus, nor was it phosphoprotein-phosphorus as usually defined. However, by mild alkaline hydrolysis of TRPR, the phosphorus was completely released in soluble organic form which was primarily inositol phosphate(s). These observations and the concomitant finding of combined fatty acids in the intact TRPR suggested that the phosphorus in this material was a constituent of a phosphoinositide. Attempts to isolate the phosphatide showed: (a) that it was not extracted by neutral solvents in batch extractions; (b) that it was extracted only very slowly by continuous extraction in a Soxhlet apparatus, and only with concomitant chemical degradation; and (c) that it was extracted readily by acidified organic solvents, *i.e.*, it appeared to be combined to the protein by a salt-like linkage. The material recovered from the latter extracts was subsequently soluble in neutral organic solvents, and appeared to be a phosphoinositide-peptide complex to which the name of phosphatido-peptides has been given (FOLCH, 1952). About 75% of the TRPR remains insoluble after acid solvent extraction, but it has lost essentially all its phosphorus.

The problem of a possible correlation between classical neurokeratin and TRPR was investigated in two ways. In the first place, TRPR was subjected to the more drastic steps followed in the preparation of neurokeratin, and it was found that phosphorus and inositol were thereby removed. In the second place, neurokeratin was prepared by the method of BLOCK (1931), and the fate of the combined inositol and phosphorus which had been recognized in the trypsin-resistant fraction was followed during the various steps. It was found that inositol and phosphorus were gradually removed, and the final product contained only traces of these substances (about 0·1% in each case). These results are compatible with the assumption that neurokeratin is derived, at least in part, from the degradation of TRPR.

EXPERIMENTAL

Analytical methods

The analytical methods used in this work, including the bioassay for inositol, have been described in previous papers from this laboratory (FOLCH, 1949; FOLCH and LEES, 1951; FOLCH *et al.*, 1951). This bioassay is inherently accurate to ±8% in our experience.

Preparation of brain trypsin-resistant protein residue (TRPR)

Extractable lipides were removed from the tissue by homogenizing it in a Waring Blender with 20 ml. of 2:1(*v*/*v*) chloroform:methanol/g. fresh tissue and collecting the insoluble residue on a

Büchner funnel (FOLCH *et al*, 1951). The tissue used was either whole beef brain, or white matter or grey matter obtained by gross dissection. The resulting residue contained all the tissue protein except proteolipides. To obtain it as a friable powder it was washed by resuspending twice in chloroform: methanol and once in acetone, and collecting each time on a Büchner funnel. After drying with acetone the residue was ground. It was washed with water* to remove the remaining acid-soluble material as follows: the powder was wetted with a minimum amount of ethanol, suspended in one hundred times its weight of water, and stirred mechanically for thirty minutes; then the residue was collected by centrifugation. The procedure was repeated with the same volume of water, and the residue was finally dried by washing with acetone. For enzymatic digestion the protein was suspended in 0·1% solution of 1–300 Trypsin (Nutritional Biochemicals Corp.) at pH 9 in the proportion of 25 ml/g. protein. This suspension was layered with toluene and incubated at 37° for two weeks, the trypsin being renewed after the first week. At the end of the digestion the pH was adjusted to 4·5 and the insoluble material collected by centrifugation. It was washed once with 0·1 N HCl, twice with water, and once with acetone. The resulting product (TRPR) was a light brown powder which was insoluble in water, dilute acid or alkali, or organic solvents. The results of analyses of several preparations of this type are given in Table 1. These residues were resistant to the action of pepsin, erepsin, and papain.

TABLE 1.—COMPOSITION OF DIFFERENT PREPARATIONS OF TRYPSIN-RESISTANT PROTEIN RESIDUE (TRPR)

Tissue	Yield mg/100 mg protein	Composition			
		Phosphorus %	Nitrogen %	Sulphur %	Inositol (all combined) %
Whole beef brain	—	1·21	—	1·43	1·6
	6·0	1·29	—	—	—
White matter	5·5	1.60	—	—	—
	5·9	1·75	10·6	1·17	2·6
	7·1	1·77	9·25	1·42	3·4
	5·4	1·79	8·42	1·50	3·4
Grey matter	5·6	0·45	10·3	1·58	0·6
	5·8	0·44	9·35	2·15	0·7

Nature of the phosphorus in TRPR; isolation of inositol phosphate

The P-containing constituents of tissues are usually divided into four main categories: acid-soluble phosphates, phosphatides, nucleic acids, and phosphoproteins. During preparation of TRPR the first two of these groups were removed. To ascertain whether or not the P remaining in this residue was in nucleic acid or phosphoprotein, some of the residue was subjected to the partition procedures of SCHNEIDER (1945) and SCHMIDT and THANNHAUSER (1945).

* FOLCH J. and MARSHALL C., unpublished.

(a) To extract nucleic acids by SCHNEIDER's technique (1945), 50 mg. of whole-brain TRPR were digested with 6 ml 5%(*w/v*) trichloroacetic acid (TCA) for 15 min at 90° and the solution brought to a final volume of 25 ml. A blank of 6 ml 5% TCA was treated in an identical way. 43% of the P of the residue was extracted, so that the P concentration of this solution was about 10 μg/ml, but the ultra-violet absorption curve, read from 245 mμ to 320 mμ against the TCA blank, had only a small peak at 270 to 280 mμ, and the total absorption at 260 mμ corresponded to a nucleic-acid P concentration of only about 0·5 μg/ml. It was clear therefore that no more than 5% of the P present could be nucleic acid P. Since there was considerable absorption at 280 mμ, most of the absorption of the extract was, in fact, due to amino acids. Therefore, much less than 5% of the P in the extract, and much less than 2% of the P in the residue, was from nucleic acids.

(b) It was found, by using the Schmidt-Thannhauser procedure (1945), that treatment with 1 N NaOH at 37° overnight released all the P from the residue as acid-soluble P in organic combination. This indicated that it was not derived from the classical type of phosphoprotein, since, in that event, the P would appear as inorganic phosphate.

(c) It thus appeared that the P in TRPR was neither nucleic acid P nor phosphoprotein P and, for further characterization, it was isolated by precipitation with lead. 3·7 g of whole brain TRPR were digested with 110 ml of 0·1 N KOH for 30 hr at 37°. After this treatment an aliquot was removed and analysed for acid-soluble P by the method of SCHMIDT and THANNHAUSER (1945). It showed that 96% of the P had been released in water-soluble form. The whole mixture was then transferred quantitatively into a centrifuge bottle with 5 ml of wash water and acidified by addition of 10·7 ml of 3 N HCl. At this stage fatty acids and other ether-soluble materials were removed by exhaustive extraction with ether, nine extractions being necessary. Residual ether was removed from the final water phase by immersing the tube in hot water. Then 40 ml of 10% TCA were added and the insoluble material was removed by centrifugation. TCA was removed from the supernatant solution by repeated extractions with ether. Upon evaporating these successive ether extracts to dryness and titrating the residues with alkali, it was found that four ether extractions sufficed for removal of all TCA.

HCl was then removed by evaporating the solution to dryness *in vacuo* at 55–60°. The residue was redissolved in a minimal amount of water, insoluble impurities were removed by centrifugation, and the supernatant fluid (about 60 to 80 ml) filtered. This filtrate was treated with 10 ml 25% neutral lead acetate and left overnight at 4° to allow complete precipitation of lead salts. These were collected by centrifugation, washed with 20 ml water, and then suspended in 60 ml water. A few drops of glacial acetic acid were added and H_2S was passed in for fifteen min to decompose the lead salts. The resulting lead sulphide suspension was centrifuged and the supernatant solution filtered several times and evaporated to dryness at 55–60° *in vacuo*. The remaining solids were washed quantitatively into a weighing bottle and dried to constant weight in a vacuum desiccator. The yield was 124·8 mg of a clear, colourless material which was easily soluble in water. Analyses of it were as follows: 15·5% P, 17·8% C, 0·7% N; the atomic C/P ratio was 3.07. It gave a strong positive Scherer reaction for inositol (SALKOWSKI, 1910). To isolate inositol, 62·5 mg were submitted to strong acid hydrolysis and further processing as described elsewhere (FOLCH, 1949). About 10 mg of crystals were obtained which were analysed as 100% inositol by bioassay.

The isolation of the organic phosphate released from TRPR by alkali was repeated with a preparation from white matter. The isolated product contained 16·0% P, 0·5% inorganic P, and 35% inositol (all combined); moles organic P/inositol = 2·6. It accounted for about half of the P in the original TRPR and 5·7% of its weight.

Study of the possible phosphatide nature of the P in TRPR

The foregoing results showed that the P in TRPR was neither nucleic acid P nor phosphoprotein P, but primarily inositol phosphate(s), and suggested that this was part of a phosphatide bound to the protein by a linkage impervious to the action of neutral solvents. In attempting to further substantiate this possibility, studies were conducted along two lines. On the one hand, TRPR was analysed for phosphatide constituents other than inositol, and, on the other, extraction of the phosphatide was attempted by a variety of procedures.

VOL 1 1956/

(a) *Analyses for other phosphatide constituents.* It had already been shown (*v.s.*) that treatment of TRPR with 0·1 N KOH for thirty hours at 37° resulted in the liberation of fatty acids. In one case in which the crude fatty acid fraction thus produced was collected and weighed, it amounted to 6·3% of the weight of the TRPR. Subsequently, aliquots of TRPR were saponified with 10 N KOH at 100° for twenty to twenty-four hours, and crude fatty acid fractions amounting to up to 20% of the weight of the original residue were obtained by ether extraction.

In addition, dilute KOH hydrolysates of TRPR were chromatographed on paper. Small amounts of amino acids were present, presumably from protein hydrolysis, but no choline or ethanolamine could be detected.

(b) *Attempts to extract phosphorus from TRPR with organic solvents.* A variety of solvents was used in attempts to extract the P-containing moiety from the TRPR both by simple batch extractions at room temperature and by boiling under reflux for a short time. Thus, petroleum ether, ethyl ether, benzene, chloroform, methanol, ethanol, and acetone were used singly and in various mixtures, but in no case was any material removed from the TRPR.

(c) *Extraction of phosphatido-peptides by acidified organic solvents.* In conjunction with these studies it was found that substantially all of the P could be removed from TRPR by the use of a chloroform: methanol: conc. HCl mixture 200:100:1 by volume (FOLCH and LEBARON, 1953). The material recovered from these extracts was soluble in neutral chloroform: methanol. It represents a new type of phosphatide to which the name phosphatido-peptides has been given. This work has been reported in summary form (FOLCH, 1952) and will be given in detail in a subsequent paper.

VOL. 1 66/57

The correlation of neurokeratin and TRPR

It was obvious from the results obtained in the work described above that TRPR was very similar to neurokeratin except for the fact that it contained a closely bound phosphoinositide which was not found in neurokeratin. This suggested that classical neurokeratin could be composed, at least in part, of products of degradation of TRPR. This possibility was explored in two ways. On the one hand, TRPR was subjected to the most drastic steps followed in the preparation of neurokeratin. On the other hand, the fate of P and inositol was followed at each step in the preparation of neurokeratin from fresh tissue by the classical procedure.

(a) *Effect of some of the steps used in the preparation of neurokeratin on the P of TRPR.* The two most drastic steps used in the preparation of neurokeratin are continuous extraction with hot ethanol for thirty days and digestion with pepsin in 0·1 N HCl.

(1) Effect of continuous extraction on TRPR. 1 g of white matter TRPR was extracted with ethanol in a Soxhlet extractor for seven weeks with frequent changes of solvent. Each successive extract was dried, and the residue weighed and analysed. The amounts of material extracted decreased with time, but extraction continued even after seven weeks. The residues were only partly soluble in chloroform: methanol, and contained inositol, organic and inorganic P, and both combined and free amino acids. At least nine different ninhydrin-positive spots were found by paper chromatography. At the end of the seven-week period, the material remaining in the extraction thimble weighed about 700 mg and contained 0·58% P and 1·3% inositol, as compared with 1·8% P and 3·4% inositol in the starting TRPR. In summary, it appeared that a major part of the P and inositol was extracted from TRPR along with a considerable amount of other material, and that the extraction was accompanied by gross chemical degradation.

This extraction could have occurred in either of two ways. It could be a physical extraction followed by degradation of the extracted material in the boiling solvent, or it could be chemical degradation of the bound phosphate moiety by the hot polar solvent followed by extraction of the degradation products. In order to test which of these hypotheses was correct, a number of experiments were carried out in which TRPR was extracted continuously with solvents of various polarities and at different temperatures. To test the effect of polarity, petroleum ether, methanol, chloroform, and ethanol were used singly or in mixtures in regular Soxhlet extractors. To test the effect of temperature, extraction with ethanol in a regular extractor (in which the temperature in the extraction chamber varied between 68° and 78°) was compared with ethanol extraction in a modified Soxhlet extractor in

which the extraction chamber was continuously cooled by tap water and the temperature was maintained at 13°. In all cases the weights of material removed in the solvents and the losses in P content of TRPR were measured. It was found that the amount of material extracted increased with increase in polarity of the solvent and with increase in the temperature in the extraction chamber. Thus petroleum ether and chloroform extracted little or nothing, a mixture of chloroform and methanol extracted a little material, and methanol or ethanol in uncooled extraction chambers extracted much more. However, nothing was extracted by ethanol when the extraction chamber was kept cooled to 13°. It was therefore concluded that the extraction of P from TRPR occurred only after prior degradation by a hot polar solvent, and that such degradation would certainly occur to a considerable degree during the preparation of neurokeratin by the classical procedure.

(2) Effect of treatment with 0·1 N HCl. The procedure used for peptic digestion in preparing neurokeratin consists of treating the tissue residue with pepsin in 0·1 N HCl for three to four weeks at room temperature. To ascertain the extent of hydrolysis of white matter TRPR under these conditions, 20·6 mg were left at room temperature in 2 ml of 0·1 N HCl for thirty-seven days, at which time 2 ml of 5% TCA were added, the mixture was centrifuged, and the supernatant solution was analysed for total and inorganic P. This revealed that 44·6% of the P was present in the solution, although less than 4% of it was inorganic phosphate; i.e. the bulk of it was presumably released as inositol phosphate. Therefore, the acid conditions of the peptic digestion used in neurokeratin preparation would remove some of the posphate moiety of TRPR.

(b) *Preparation of neurokeratin.* In order to allow the removal of P and inositol from the tissue residue during actual preparation of neurokeratin, 490 g of cattle brain were treated by the procedure given by BLOCK (1931). The material obtained after extraction in a Soxhlet extractor with 95% ethanol, termed "neurokeratinogen" for convenience, amounted to 33·0 g and had a P content of 0·43% and an inositol content of 0·08%. However, this product still contained some of the acid-soluble constituents of brain. By washing it twice with 0·1 N NCl its P content was reduced to 0·17% and its inositol content to 0·06%. Portions of this "neurokeratinogen" were digested with pepsin of trypsin, the residues being analysed for P and inositol. The pepsin-digested residue was then carried through the rest of the procedure described by BLOCK to yield neurokeratin itself. After digestion of "neurokeratinogen" with pepsin the residue represented 7·3% of the original material and its inositol content represented only 17·5% of that originally present in "neurokeratinogen." After digestion of "neurokeratinogen" with trypsin there remained only 10·3% of the solids, but 41·2% of the original inositol was present in it. When the pepsin-digested residue was subsequently treated with trypsin to yield neurokeratin, there was an additional loss of weight and inositol so that the neurokeratin represented 5·6% of the "neurokeratinogen" and contained 10·1% of its inositol. The final analyses were: pepsin-digested "neurokeratinogen", 0·20% P and 0·12% inositol; trypsin-digested neurokeratinogen, 0·16% P and 0·20% inositol; and neurokeratin, 0·13% P and 0·09% inositol. It thus appeared from the foregoing results that P and inositol were gradually removed from the protein residue during preparation of neurokeratin.

DISCUSSION

The protein-bound inositol in TRPR represents a new type of combined inositol. In brain, the only previously described form of combined inositol is the phosphatide, diphosphoinositide (FOLCH, 1949). Inositol phosphatides have been described from other sources but none has been chemically bound to protein. It is possible that the inositol phosphate of TRPR is bound in the same basic chemical structure that occurs in brain diphosphoinositide, but the present information is inconclusive. Further work is now in progress.

The P in this fraction, being insoluble in neutral lipide solvents and releasing organically combined phosphorus on mild treatment with alkali, represents a fifth category of tissue phosphorus compounds, since it cannot be classified under any of the usual ones; acid-soluble phosphorus, free phosphatides, nucleic acids, or phosphoproteins as usually defined. Of practical importance is the behaviour of this bound phosphorus in the partition of nervous tissue phosphorus compounds by the methods

VOL 1 1956/

of SCHMIDT and THANNHAUSER (1945) and of SCHNEIDER (1945). In the method of SCHMIDT and THANNHAUSER (1945), as previously reported (FOLCH and LEBARON, 1951), phosphorus from TRPR appears quantitatively as organically combined phosphorus in the ribonucleic acid fraction, a fact which invalidates data for ribonucleic acid obtained by phosphorus estimation. In the method of SCHNEIDER (1945), the phosphorus of TRPR is distributed between the nucleic acid and the phosphoprotein fractions and would render meaningless the phosphorus values in these fractions. LOGAN *et al.* (1952) have taken this into account in their method for determining nucleic acids in nervous tissue. A simpler method has been devised in this laboratory in which the phosphorus in TRPR is extracted with uncombined phosphatides by the use of an acidified solvent.

This phosphorus in TRPR may prove to have an important metabolic role. Recent work with radioactive phosphorus has demonstrated a relatively high exchange rate for a protein-bound phosphate which is probably the fraction described here (SAMUELS *et al.*, 1951; STRICKLAND, 1952; DELUCA *et al.*, 1953; FINDLAY *et al.*, 1954).

TABLE 2.—COMPOSITION OF PREPARATIONS OF NEUROKERATIN AND OF ITS POSSIBLE PRECURSORS

Preparation	Constituent				
	Carbon %	Hydrogen %	Nitrogen %	Sulphur %	Phosphorus %
Neurokeratin					
EWALD and KÜHNE (1874–77)	—	—	Present	2.93	—
KÜHNE and CHITTENDEN (1890)	56·1–58·5	7·3–8·0	11·5–14·3	1·63–2·24	"None"
ARGIRIS (1907)	56·6	7·4–7·5	14·2	2·2–2·3	—
NELSON (1916)	54·9	7·3	13·2	1·4	"Entirely absent"
BLOCK (1931)	55·5	6·96	14·1	1·98	0·00
BLOCK (1937)	55·2	7·26	13·32	1·62	—
This paper	—	—	11·03	1·75	0·135
Proteolipide protein (FOLCH and LEES, 1951)	—	—	14·0	1·76	<0·1
Whole-brain trypsin-resistant protein residue (TRPR)	—	—	10·0	1·43	1·25

Concerning the relation between TRPR and neurokeratin, the following can be said. Neurokeratin and TRPR are alike in many respects including chemical composition (see Table 2). The main difference between them is the presence in the latter of significant and consistent amounts of combined phosphorus and inositol, both of which are essentially absent in the former. However, during the preparation of neurokeratin by classical methods, drastic procedures are used which have been found to remove most of the combined phosphorus and inositol from the tissue. Indeed, some of the earliest versions of the method included a final extraction of "nuclein" with dilute alkali which would complete this removal. Thus it appears that TRPR can be a precursor of neurokeratin. As has already been reported (FOLCH and LEES, 1951),

proteolipide protein also has the necessary characteristics to be classified as a neurokeratin fraction. Proteolipide would not be included in TRPR since it is removed in the original lipide extraction, but, in the classical preparation of neurokeratin, proteolipide would be split and the protein moiety would be found in the final residue. In summary, classical neurokeratin is most likely a combined degradation product of proteolipides and TRPR. The variation in the composition of neurokeratin from preparation to preparation (Table 2) could easily be explained by small variations in the procedures of preparation which would result in different degrees of degradation.

SUMMARY

A trypsin-resistant protein fraction has been prepared from brain tissue by removal of lipides extractable with neutral solvents and subsequent digestion with trypsin. This fraction contains a moiety extractable only by acidic solvents which consists of inositol phosphate(s) and fatty acids, indicating that it is a phosphoinositide bound by a salt-like linkage. It represents a new type of combined inositol in brain tissue. Neurokeratin was prepared by the classical method and found to contain small residual amounts of inositol and phosphorus. The relation of the trypsin-resistant protein residue to neurokeratin and its behaviour in the partition of tissue phosphorus components were discussed.

REFERENCES

ARGIRIS A. (1907) *Hoppe-Seyl. Z.* **54,** 86.
BLOCK R. J. (1931) *J. Biol. Chem.* **94,** 647.
BLOCK R. J. (1937) *J. Biol. Chem.* **121,** 761.
BLOCK R. J. (1951) *Arch. Biochem. and Biophys.* **31,** 266.
DELUCA H. A., ROSSITER R. J., and STRICKLAND K. P. (1953) *Biochem. J.* **55,** 193.
EWALD A. and KÜHNE W. (1874–77) *Verhandl. Naturhist-Med.* **1,** 457.
FINDLAY M., STRICKLAND K. P., and ROSSITER R. J. (1954) *Canad. J. Biochem. and Physiol.* **32,** 504.
FOLCH J. (1949) *J. Biol. Chem.* **177,** 505.
FOLCH J. (1952) in MCELROY W. D. and GLASS B. *Phosphorus Metabolism* Johns Hopkins Press, Baltimore, **2,** 195.
FOLCH J., ASCOLI I., LEES M., MEATH J. A., and LEBARON F. N. (1951) *J. Biol. Chem.* **191,** 833.
FOLCH J. and LEBARON F. N. (1951) *Fed. Proc.* **10,** 183.
FOLCH J. and LEBARON F. N. (1953) *Fed. Proc.* **12,** 203.
FOLCH J. and LEES M. (1951) *J. Biol. Chem.* **191,** 807.
FOLCH J. and WOOLLEY D. W. (1942) *J. Biol. Chem.* **142,** 963.
KÜHNE W. and CHITTENDEN R. H. (1890) *Z. für Biol.* **26,** 291.
LOGAN J. E., MANNELL W. A., and ROSSITER R. J. (1952) *Biochem. J.* **51,** 470, 480.
NELSON B. E. (1916) *J. Amer. Chem. Soc.* **38,** 2558.
SALKOWSKI E. (1910) *Hoppe-Seyl. Z.* **69,** 478.
SAMUELS A. J., BOYARSKY L. L., GERARD R. W., LIBET B., and BRUST M. (1951) *Amer. J. Physiol.* **164,** 1.
SCHMIDT G. and THANNHAUSER S. J. (1945) *J. Biol. Chem.* **161,** 83.
SCHNEIDER W. C. (1945) *J. Biol. Chem.* **161,** 293.
STRICKLAND K. P. (1952) *Canad. J. Med. Sci.* **30,** 484.
THUDICHUM J. L. W. (1901) *Die Chemische Konstitution des Gehirns des Menschen und der Tiere* (F. Pietzcker, Tübingen).

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 109 to 118. Pergamon Press Ltd., London

METABOLIC PROPERTIES OF CEREBRAL TISSUES MODIFIED BY NEOPLASIA AND BY FREEZING

J. B. BRIERLEY and H. MCILWAIN
Departments of Neuropathology and Biochemistry, Institute of Psychiatry
British Postgraduate Medical Federation, University of London
Maudsley Hospital, London

(*Received* 8 *June* 1956)

NEOPLASTIC cerebral tissues have received much less metabolic study than have those from several other parts of the body. Aspects of the resting metabolism of cerebral tumours have been reported by VICTOR and WOLF (1937) and during the course of the present investigation by HELLER and ELLIOTT (1955). However, an especial interest of such tissues lies in their derivation from components of an excitable organ whose level of metabolism changes markedly with its level of functional activity. Techniques for studying metabolic responses to excitation in separated cerebral tissues have been developed (MCILWAIN, 1951, 1955) and are here applied to neoplasms obtained during neurosurgical operations. A preliminary report has been made (MCILWAIN, 1954).

Results are also presented of a cognate study of cerebral tissues modified experimentally. Portions of the animal brain were exposed to low temperatures, and samples were examined metabolically after various survival periods, for each of which the corresponding histological appearances were known (BRIERLEY, 1956). In each case comparison has been made with the metabolic behaviour of normal cerebral tissues from the same animal species.

EXPERIMENTAL

Neurosurgical specimens. Immediately after excision, the material selected for metabolic study was placed in a small polythene container immersed in ice-water and brought to the laboratory (MCILWAIN, AYRES, and FORDA, 1952). Here preparations for the experiment had already been made and the tissue was sliced or chopped, weighed, and placed in electrode vessels (see AYRES and MCILWAIN, 1953 for details of electrical apparatus and procedure). Experiments were done on the tissues as described below for rat and rabbit tissues, except that vessels of 15-ml capacity were used.

Pieces of tissue adjacent to each of the samples listed in Table 1 were examined histologically. A description of each follows.

(1) This was a sample of apparently normal cortex adjacent to an astrocytoma. (2) A glioblastoma of magnocellular structure and moderate cell density. Its vascularity was fairly low, but necrosis was not seen. (3) An area of frontal cortex and subjacent white matter showing a moderate diffuse fibrous gliosis. (4) A capillary haemangioblastoma of the cerebellum showing areas of compactly arranged capillaries with proliferating endothelial cells. In some areas the vessels were sinusoidal and included some very large channels. (5) A capillary haemangioblastoma of the cerebellum, made up of compactly arranged capillaries with proliferating endothelial cells. The vessels were embedded in a dense fibrous and collagenous matrix. No nerve or glial cells were seen. (6) A well differentiated cystic astrocytoma in which the cells were largely protoplasmic astrocytes. The cysts were variable in size and the tumour was well vascularized. (7) An oligodendroglioma consisting of uniform nuclei not densely packed together. There was very little interstitial connective tissue and no perinuclear halo formation. (8) A glioblastoma multiforme consisting of rather loosely arranged glioblastic tissue. The cells were of varied shapes and sizes and included some giant forms. Vascularity was poor and some areas of necrosis were present.

Frozen Tissues. A midline skin incision was made over the skulls of rats or rabbits anaesthetized

with Nembutal. A copper tube, open at both ends and of internal diameter 8 mm, was mounted on the exposed intact skull within a grease seal. Liquid oxygen was poured into this tube and allowed to boil off. Freezing times varied from 10 (mild) to 25 sec (severe) and produced lesions ranging from 4 × 8 mm to 5·5 × 12 mm. Recovery of consciousness occurred in 1 to 3 hr, except after some severe lesions, when it was delayed for up to a further 12 hr. The range of permitted survival periods was from 12 to 28 days for rats and from 28 to 63 days for rabbits.

To obtain the tissue, rats were stunned by a blow on the neck, exsanguinated, and the skull opened. Rabbits (average weight, 2·9 kg; average brain weight, 8·5 g) were killed by injection of air into an ear vein, exsanguinated, and the skull vault separated from the base with bone forceps. The

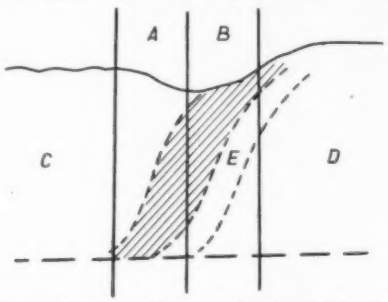


FIG. 1—Diagram of margin of frozen lesion in rabbit cerebral cortex. *A* = inner and *B* = outer zones of margin, *C* = centre of lesion, *D* = normal cortex, and *E* = zone of altered nerve cells. Zone of gliosis shaded.

lesion, usually adherent to the vault and requiring separation with a scapel, appeared as a sunken and discoloured area. A block of tissue containing the lesion was carefully excised, the normal tissue dissected off, and the affected tissue then divided up as required for individual experiments.

The normal cortical grey matter had disappeared within the treated area and the changed tissue overlay the subcortical white matter. Some of the latter was included in the samples from one animal (rabbit, survival 49 days). The term margin (Fig. 1) is used for the narrow peripheral zone of the lesion, lying between the junction with normal cortex and the brownish central part. Samples were also taken of cortex just outside the lesion or from the contralateral cortex for comparison with the lesion itself.

Histological appearances. Histological examination was confined to rabbit cerebral tissues (28–63 days). At 28 days the lesion was seen as a sharply defined saucer-like defect filled with compound granular corpuscles (loaded with the products of nerve cell and myelin breakdown). Strands of proliferating blood vessels and fibroblasts invaded this central zone from its floor. At the margins of the lesion was a strip of large proliferating fibrous astrocytes (Fig. 1). These were separated from the necrotic centre by a band of new connective tissue and from the normal cortex by a narrow line of shrunken, hyperchromatic, and presumably nonviable nerve cells. The necrotic centre of the lesion contained no nerve cells and very few recognizable astrocytes or microglia.

From one month onwards there was relatively little change in the histological appearances of the lesion. In the central zone, the phagocytes seemed unchanged at 63 days, and the only change in the marginal zone was a variable increase in the density of the connective tissue and astrocyte layers.

The marginal zone was divided into outer and inner portions in certain experiments (Table 3). From Fig. 1 it can be seen that the inner zone would contain more connective tissue, less astrocytes and nerve cells (if any) than the outer zone in which proliferating astrocytes, a little connective tissue, some damaged nerve cells, and a small proportion of normal cortex would be found.

Metabolic experiments. The dissected samples were weighed on a torsion balance (sensitivity ±0·1 mg) and portions of 10–20 mg prepared as prisms 0·25 × 0·25 × 1–2 mm by the mechanical chopper of MCILWAIN and BUDDLE (1953). The chopped tissue was transferred to 1 ml of fluid in a manometric electrode vessel of about 4 ml capacity (MCILWAIN and AYRES, 1953), dispersed, paper soaked in 5 N NaOH added for absorption of CO_2, and the vessel was fitted to its manometer and oxygen was passed through it for 3–5 min. It was then shaken in a water-bath maintained at 37°; this point in the experiment was reached 30–40 min after the death of the animal. Readings of

oxygen pressure were taken every 5 min during an experiment of total duration 2–3 hr divided into four successive periods each of 30–45 min. During the first and last of these, no impulses were applied. During the second, condenser pulses of time-constant 0·3–0·4 msec were applied to some vessels at 5–6 V and during the third at 10–12 V. Specimens were taken out promptly at the end of the experiment for determination of their lactic-acid content.

Salines employed were those of McIlwain (1951); glucose was used as a substrate at a concentration of 10 mM, and sodium glutamate at a concentration of 20 mM. Serum was prepared from rabbit blood by removing bicarbonates according to Field (1948) and adding phosphate (final concentration 20 mM) and glucose (final concentration 10 mM).

RESULTS

Neurosurgical Specimens

The metabolism of normal human cerebral tissues has been examined previously under conditions closely similar to those of the present study (McIlwain, 1953*b*). The tissues were found to be capable of metabolic response to applied electrical pulses, as is shown by relevant results summarized in Table 1. Here the two main energy-yielding reactions of the tissues have been measured, i.e. respiration and the formation

Table 1.—Metabolism of human cerebral tissues

Number and nature of specimen	Respiratory rate (μmoles O_2/g/hr) in successive periods				Lactic acid (μmoles/g/exp)
Normal (see McIlwain, 1953): cerebral cortex	41 ± 8 (40)	*64*	**81**	40	30, **56**
Normal (see McIlwain, 1953): subcortical white matter	25 ± 6 (5)	34	39 ± 8 (4)	24	21, **37**
(1) Cerebral cortex adjacent to astrocytoma	43	43	43	43	— —
	39	*56*	*56*	45	— —
	40	**64**	**64**	45	— —
(2) Glioma: immediately adjacent cortex and white matter[1]	40	40	40	40	60
	42	*45*	**47**	41	**69**
(2) Glioma: centre of tumour[2]	1	2	0	1	15
	2	*1*	**2**	2	**14**
(3) Mixed grey and white matter of cerebral cortex showing gliosis	29	29	29	29	63
	26	*38*	**55**	33	**77**
	28	*45*	**61**	31	**68**
(4) Cerebellar haemangioblastoma: Outer tissue of tumour	9	9	9	9	28
	17	*18*	**18**	16	**39**
(4) Cerebellar haemangioblastoma: Core of tumour	4	4	4	4	23
	6	*4*	**4**	5	**28**
(5) Cerebellar haemangioblastoma	7	7	7	7	70
	13	*15*	**15**	14	68
	8	*7*	**7**	7	55
(6) Cystic astrocytoma	8	8	8	8	53
	10	*10*	**10**	7	51
(7) Oligodendroglioma: more normal parts[3]	3	4	4	4	28
	5	**6**	**4**	4	**23**
(7) Oligodendroglioma: less normal parts[4]	2	4	4	2	24
	2	**3**	**3**	1	**25**
(8) Glioblastoma multiforme[5]	2	*2*	**2**	2	11

Specimens are more fully described on page 109. Experimental periods were of 30 to 40 min. Respiratory values in italics: during application of pulses at 10 V, time-constant 0·4 msec, at 100/sec; in bold type: pulses at 18 V but otherwise the same. Lactic acid: values in bold type, during application of pulses as indicated in the columns giving respiratory rate. Medium: glucose-phosphate saline. In addition, replacement of glucose by 20 mM succinate afforded the following respiratory rates (notation as in Table) [1]: 70 *66*, **66**, 54; [2]: 2, *2*, **2**, 1; [3]: 2, **4**, **4**, 4; [4]: 1, **2**, **2**, 2; [5]: 4, 2, **2**, 1.

of lactic acid from glucose. It can be seen that pulses nearly doubled the respiratory rate and the accumulation of lactic acid. This occurred in both the cerebral cortex and in the subcortical white matter.

Almost all the specimens obtained from or near to neoplastic tissue showed clearly recognizable metabolic abnormalities. The exception was specimen (1), adjacent to an astrocytoma and showing respiratory rate and metabolic response within the normal range. By contrast, cortex adjacent to the glioma of specimen (2), although normal in respiratory rate, was abnormal in showing only a small respiratory response to electrical pulses and in having a high rate of glycolysis. The centre of this glioma (of magnocellular type and fairly low vascularity) was, however, very different metabolically in that both respiratory and glycolytic rates were low and there was no metabolic response to pulses. All characteristics were closer to normal in specimen (3), which from histological examination appeared to be an area of moderately severe gliosis. The chief metabolic abnormality lay in its high rate of glycolysis.

The metabolism of the remaining specimens was similar in that the respiratory rates were abnormally low and there was no ability to respond to pulses by increased respiration. Of this group, the two haemangioblastomata (4) and (5) showed the greater respiratory rates and specimen (4) (a capillary haemangioblastoma showing active proliferation of endothelial cells) showed a small glycolytic response to pulses. The astrocytoma (6) (cystic and consisting of protoplasmic astrocytes) had a high glycolytic rate unaltered by pulses. The oligodendroglioma (7) might be said to possess a normal glycolytic rate except that, in normal tissue, such a rate would not persist when the respiration was as low as it was in this specimen. There was virtually no respiration and glycolysis in the glioblastoma (8) (cells of very varied shape and size and including some giant cells).

Experimental Lesions

Preliminary experiments. The brains of rats in the first two experiments (Table 2) were subjected to only a brief freezing treatment, and little change could be seen by naked-eye in the cerebral cortex. Metabolically, the tissue at the site of chilling was normal. In the third group of rats, subjected to more prolonged exposure to liquid oxygen, there was visible change in the cortex after 12 days. The respiratory rate of the lesion was low, but responded proportionately to applied impulses. At this stage in the experiments it became evident that if parts of the lesions were to be studied, even in the smallest apparatus available, either many rats or a larger animal would be needed. Rabbits were chosen.

The brain of the rabbit of experiment 4 received mild cooling and yielded a small and only slightly discoloured lesion. The central, most affected part was removed for the experiment, but included some subcortical white matter. Respiration was low, but response to pulses and glycolysis was normal. From experiment 5 onwards freezing was severe and resulted in lesions of the type described earlier (under "frozen tissue"). Experiment 5 was carried out with tissue samples from the whole of the affected area. Respiration had been greatly reduced, but respiratory responses were maintained.

In Table 3, the metabolic behaviour of parts of the lesion is compared with that of adjacent normal samples from cortex, subcortical white matter, and dura, which will first be described.

VOL
1
1956/

TABLE 2.—METABOLISM OF MILDLY TREATED RAT AND RABBIT TISSUES

Experiment	Species	Treatment	Description of specimen	Respiratory rate (μmole O_2/g/hr) during successive periods				Lactic acid accumulation (μmole/g/exp.)
				1st	2nd	3rd	4th	
1a	Rat	—	Contralateral to 1b	68	*80*	**102**	76	**112**
1b	Rats (2)	Mild freezing 14 days previously	Slight interruption of cortical vessels; no depression or scar.	68	*98*	**106**	80	**89**
2a	Rat	—	Contralateral to 2b	66	*78*	**85**	68	**83**
2b	Rats (2)	As 1b, 28 days previously	As 1b	65	*91*	**101**	68	**115**
3a	Rat	—	Contralateral to 3b	75	75	78	75	100
3b	Rats (3)	Severe freezing 12 days previously	Sunken patch; cortical vessels interrupted and in two cases cortex necrotic	72	*96*	**112**	70	**138**
4a	Rabbit	—	Contralateral to 4b	57	56	61	56	*98*
4b	Rabbit	Mild freezing 42 days previously	Sunken area 6 × 3 mm slightly discoloured	58	*96*	**104**	59	**117**
5a	Rabbit	—	Contralateral to 5b	46	52	52	50	*53*
5b	Rabbit	Severe freezing 28 days previously	Sunken area 4 × 8 mm	46	*62*	**92**	40	**84**
				30	*44*	**56**	32	*105*
				52	52	52	52	—
				48	**95**	**95**	46	—
				4·5	**6**	**10**	4·5	—
				3	**8**	**5**	6	—

Experimental periods were normally of 30 min, commencing 20–30 min after death of the animal. Respiratory values in italics: during application of pulses of peak potential 5–6 V, time-constant 0·4 msec, 100/sec; in bold type: with pulses at 10–12 V and otherwise similar. Values for lactic acid: in bold type in vessels to which impulses were applied (normally at both voltages, as indicated in respiratory columns). Medium: glucose-phosphate saline.

TABLE 3.—METABOLISM OF NORMAL AND TREATED RABBIT CEREBRAL TISSUES

Days after treatment	Tissue	Additions to media	Respiratory rate (μmole/g/hr) during successive periods				Lactic acid (μmole/g/exp)
			1st	2nd	3rd	4th	
0–63	Grey matter of cerebral cortex beyond lesion or contralaterally placed	None	48	50	49	48	52
		None	46	*58*	**84**	47	**74**
0–63	Grey matter of cerebral cortex beyond lesion or contralaterally placed	Glutamate	92	65	60	47	—
0–63	Grey matter of cerebral cortex beyond lesion or contralaterally placed	Glutamate	80	*62*	**63**	33	—
0–63	Grey matter of cerebral cortex beyond lesion or contralaterally placed	Serum	92	90	85	80	—
0–63	Grey matter of cerebral cortex beyond lesion or contralaterally placed	Serum	90	*98*	**120**	76	—
0–63	Subcortical white matter	None	16	16	16	16	29
0–63	Subcortical white matter	None	14	*16*	**24**	15	**40**
0–63	Subcortical white matter	Glutamate	15	15	14	14	24
0–63	Subcortical white matter	Glutamate	17	*17*	**21**	20	**25**
0–63	Subcortical white matter	Serum	29	29	28	26	88
0–63	Subcortical white matter	Serum	31	*26*	**26**	22	**87**
0–63	Dura	None or glutamate	**0**	**0**	**0**	**0**	16
0–63	Dura	None or glutamate	**0**	*0*	**0**	**0**	**12**
0–63	Dura	Serum	18	13	6	**0**	45
0–63	Dura	Serum	20	*14*	**8**	6	**49**
49	Margin of lesion with subcortical white matter	None	22	22	17	15	34
49	Margin of lesion with subcortical white matter	None	18	*15*	**30**	8	**44**
63	Margin (no white matter)	Serum	37	40	40	36	—
63	Margin (no white matter)	Serum	30	*46*	**46**	36	—
49	Outer margin (no white matter)	None	10	*32*	**45**	36	**53**
56	Outer margin (no white matter)	None	25	*32*	**39**	31	**47**
58	Outer margin (no white matter)	Glutamate	36	42	26	21	—
58	Outer margin (no white matter)	Glutamate	40	*44*	**44**	23	—
49	Inner margin (no white matter)	None	3	**0**	**0**	**0**	18
49	Inner margin (no white matter)	None	4	*4*	**0**	**0**	**23**
56	Inner margin (no white matter)	None	6	**0**	**0**	**0**	17
49	Centre of lesion	None	**0**	*8*	**0**	**0**	**28**

All media contained glucose and were buffered with phosphate; additional materials are named above. Pulses and expression of results, as in Table 2.

VOL 1 1956/

Normal tissues. The respiratory rate of rabbit cerebral cortex in the glucose saline used in the present experiments approximated to that previously reported for slices or suspensions in similar media (ELLIOTT 1948; MCILWAIN, 1953a), but was lower than those quoted for slices of the same tissue in media with lowered concentrations of calcium salts or with bicarbonate or other additions (KREBS, 1950). In serum, respiration was markedly higher but less stable; with glutamate as substrate, initial rates in saline were almost as high as in serum, but the subsequent decrease was greater. Electrical pulses had their greatest effect on tissues in glucose saline and least on tissues in glutamate saline. Subcortical white matter respired in glucose saline at about a third of the rate of cerebral cortex; the rate was a little increased by glutamate and rather more so by serum, but even so remained at about a third that of the cortex in the corresponding medium. Respiratory and glycolytic responses to pulses were evident in the glucose medium, but not in the others. The dura, as would be expected, was least active metabolically, and in saline did not respire appreciably with glucose or glucose plus glutamate as substrates. In serum, initial respiration was at about one-third of the rate of cerebral cortex, but showed no response to applied pulses. Glycolysis was appreciable in the simpler media, and was increased by serum but not by applied pulses.

Frozen tissues. The centre of the lesion respired little if at all, but had about half the glycolytic rate of the cerebral cortex. Of the normal tissues it was thus akin to dura. The margin of the lesion had respiratory and glycolytic rates which were higher than those of the subcortical white matter with which it might have been admixed. The rates were therefore judged to be a property of the changed tissue. They remained less than those of normal grey matter. Response to pulses was seen both in glucose saline and in serum. When the outer and inner part of the margin were examined separately, the inner part showed little respiration and no clear response to impulses. The outer part had the properties described above for the whole margin of the lesion.

DISCUSSION

Of major significance in the present results is the manner in which metabolic response to electrical pulses varies in the different specimens examined. In the neurosurgical material, an increased susceptibility to pulses was never clearly demonstrated. Taken collectively, the tissues from rats subjected to local freezing also showed no increased susceptibility.

On the other hand, a decreased metabolic response is well established both for the neoplasms and in the experimentally modified tissue. No neoplasm gave an appreciable response to pulses, and in one of the two instances examined (specimen 2, Table 1) tissue adjacent to the neoplasm proper was also affected in the same way. Respiratory and glycolytic responses were, in general, lost together. In specimen 3, Table 1 (area of gliosis), an almost normal respiratory response was associated with a small glycolytic response, but here glycolysis was already high before pulses were applied.

The experimentally treated rat and rabbit tissues showed a gradation in the degree of metabolic impairment from minimum values at the centre of the most severe lesion to more nearly normal values in the marginal and adjacent zones of the mildest lesions. The neoplasms can also be arranged in such a sequence. The lowest respiratory rates occurred in the two glioblastomata multiforme (specimens 2 and 8, Table 1); the

two zones of the oligodendroglioma were a little higher (7) and higher again, the two blood-vessel tumours (specimens 4 and 5), and the cystic astrocytoma (6). The area of gliosed grey and white matter (3) respired at an appreciably lower rate than the cortex (1) adjacent to an astrocytoma, which respired at a rate within normal limits.

Comparison between the present series of tumours and those of VICTOR and WOLF (1937) and HELLER and ELLIOTT (1955) reveals several points of interest. In the first place, the present series includes a haemangioma and a haemangioblastoma, tumours derived from mesodermal tissue. Their respiration is only just greater than that of the gliomas and it shows virtually no response to pulses. (Lactic acid formation may be increased in the haemangioblastoma.) Such mesodermal tumours can be regarded as comparable to the dura mater of the rabbit and the central zone of the frozen lesion, which contains no nerve cells, a minimum of glia, and a heavy proliferation of mesodermal elements. The respiration of these two rabbit samples was negligible, but it must be recorded that the inclusion of some cerebellar tissue in the two vascular tumours was probably unavoidable.

Among the gliomata (specimens 2, 6, 7, and 8 of Table 1), respiratory rates were very low and response to pulses virtually absent. This applied also to the central region of the oligodendroglioma (7), in contrast to the findings of the other workers. In glycolysis, however, this specimen was intermediate between the very divergent values previously reported.

Thus from the present results, together with those of VICTOR and WOLF (1937) and HELLER and ELLIOTT (1955), it does not seem possible to ascribe distinctive metabolic characteristics (on a wet-weight basis) to any one type of neoplasm, whether of mesodermal or ectodermal origin.

HELLER and ELLIOTT (1955) have attempted to deduce metabolic characteristics for individual cell types from the metabolic behaviour of neoplasms and normal cerebral tissues and from the cellular densities of both. Thus, a comparison of cerebellar cortex with corpus callosum in respect of oxygen uptake per 10^6 nuclei, led to the conclusion that "non-neuronal, presumably glial, cells in the brain can therefore respire considerably more rapidly than some neurons." However, LOWRY, ROBERTS, LERNER, WU, FARR, and ALBERS (1954) have shown, in the rabbit Ammon's horn, that the dendritic processes of nerve cells are probably responsible for a great part of the metabolism of the structure. For the cerebellum this would imply that the outer (molecular) cell-poor but dendrite-rich layer would be more active than the highly cellular inner (granular) layer. The role of oligodendroglia in the metabolism of the corpus callosum was estimated by HELLER and ELLIOTT from the oxygen-consumption figures for the oligodendroglioma. Substantially lower values were obtained for a tumour of this type in the present series. Further, it may be unwise to ascribe to the axones of the corpus callosum the low oxygen-consumption figures for peripheral nerve without making corrections for the appreciable connective tissue (endo- and peri-neurium) elements of the latter.

The series of glial tumours of HELLER and ELLIOTT (1955) and of the present authors include both primitive (glioblastoma) and more differentiated (astrocytoma) forms, and it it noticeable that the two groups do not differ significantly in oxygen consumption. Response to pulses is virtually absent in both, while glycolysis is increased in both but to a greater extent in the astrocytoma.

VOL
1
1956/

Thus we feel that the conclusions of HELLER and ELLIOTT, quoted above, should be modified for the following reasons:

(1) Different neuronal and glial cells differ greatly in the volume of cytoplasm associated with one nucleus, and much of the neuronal cytoplasm lies as dendrites some distance from the nucleus and so may not be included in the same specimen.

(2) During development, the metabolic characteristics of the brain can change considerably after its nuclear count has become maximal. Concomitant changes in the metabolic response to electrical pulses also occur (GREENGARD and MCILWAIN, 1955). We believe there is still inadequate justification for assuming that the metabolism of a given cell-type is the same in such different situations as normal and neoplastic tissues.

(3) The metabolic conditions of Table 1 and of the previous studies do not necessarily exhibit the full metabolic activities of different tissues, whose relative activities may change with change of media (Table 3).

To understand the way in which the tumours can act as centres of functional disturbances would necessitate a wider study than has now been undertaken, but two relevant biochemical abnormalities have been seen. The first is the excessive production of lactic acid, which has been observed in other neoplasms (WARBURG, 1930). Secondly, instances have been found in which tissue, not only in the tumour but also surrounding it, was abnormal metabolically and abnormal in its response to electrical pulses. Indeed, normal behaviour in all these respects—respiration, glycolysis, and respiratory and glycolytic response to electrical pulses—was found only in tissue with its normal complement of nerve cells and in which increase in glial or mesodermal elements had not occurred.

SUMMARY

1. Oxygen consumption and lactic acid formation and their response to electrical pulses have been studied in normal human cerebral tissues, in cerebral tumours, and in rat and rabbit cerebral cortex modified by freezing.

2. Applied pulses caused an increase of about 60% in oxygen consumption and of up to 90% in lactic acid formation in normal human cerebral tissues.

3. The oxygen consumption of all the tumourous tissues (mesodermal and ectodermal) was very low and response to pulses absent or very small.

4. Lactic-acid formation in tumours ranged from twice the normal figure in a cerebellar haemangioblastoma to half normal in a glioblastoma multiforme.

5. In the frozen animal cortex, depression of respiration and glycolysis and of their response to pulses was greater in the centre of the lesion, where mesodermal elements predominated.

6. Although increased susceptibility to electrical pulses was not seen in neoplastic tissues, the other metabolic abnormalities found there and in some cases in surrounding tissue offer a basis for the action of tumours as centres of functional disturbance.

Acknowledgements—We are greatly indebted to Mr. MURRAY FALCONER of the Guy's-Maudsley Neurosurgical Unit, for the specimens of human cerebral tissues; and to the Board of Governors of the Bethlem Royal Hospital and Maudsley Hospital for their allocation of research funds in support of these studies.

REFERENCES

AYRES P. J. W. and MCILWAIN H. (1953) *Biochem. J.* **55,** 607.
BRIERLEY J.B. (1956) in the press.
ELLIOTT K. A. C. (1948) *J. Neurophysiol.* **11,** 473.
FIELD J. (1948) *Methods in Medical Research* **1,** 289.
GREENGARD P. and MCILWAIN H. (1955) *Neurochem. Sympos.* **1,** 251.
HELLER I. H. and ELLIOTT K. A. C. (1955) *Canad. J. Biochem. Physiol.* **33,** 395.
KREBS H. A. (1950) *Biochim. Biophys. Acta.* **4,** 249.
LOWRY O. H., ROBERTS N. R., LERNER K. Y., WU M-L., FARR A. L., and ALBERS R. W. (1954) *J. Biol. Chem.* **207,** 39.
MCILWAIN H. (1951) *Biochem. J.* **49,** 382.
MCILWAIN H. (1953a) *Biochem. J.* **55,** 618.
MCILWAIN H. (1953b) *J. Neurol. Neurosurg. Psychiat.* **16,** 257.
MCILWAIN H. (1954) *Arch. Neurol. Psychiat.* **71,** 488.
MCILWAIN H. (1955) *Biochemistry and the Central Nervous System,* Churchill, London.
MCILWAIN H., AYRES P. J. W., and FORDA O. (1952) *J. ment. Sci.* **98,** 265.
MCILWAIN H. and BUDDLE H. L. (1953) *Biochem. J.* **53,** 412.
VICTOR J. V. and WOLF A. (1937) *Proc. Assoc. Res. nerv. ment. Dis.* **16,** 44.
WARBURG O. (1930) *The Metabolism of Tumours,* trans. F. Dickens. Constable, London.

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 119–125, Pergamon Press Ltd., London.

HISTOCHEMISTRY AND CHARACTERIZATION OF HYALURONIC ACID IN AXONS OF PERIPHERAL NERVE*

L. G. ABOOD and S. K. ABUL-HAJ†
Division of Psychiatry, University of Illinois College of Medicine

(*Received* 10 *July* 1956)

CATIONIC adsorption, or accumulation, within tissues has been attributed to many substances, including proteins (see LING (1955) for discussion), phospholipids (FOLCH, 1952), soluble constituents, such as polyphosphates (STEINBACH, 1950), chondroitin sulphate (MEYER and RAPPORT, 1951), and to ground substance colloids (JOSEPH, ENGEL, and CATCHPOLE, 1952). In view of certain difficulties concerning the hypothesis that the internal potassium concentration exists in a free form and is maintained by a "sodium pump mechanism," LING (1955) has postulated that "fixed charges" within the nerve are responsible for the selective accumulation of potassium. Studies of the energy requirements of nervous tissue during excitation have suggested that "high-energy phosphates" are primarily concerned with the maintenance of structural relationships essential for activity and, presumably, are not specifically involved in a sodium pumping mechanism (ABOOD and GOLDMAN, 1956). In an effort to determine whether nucleic acids, or the microsomes of which they are an integral part, are involved in ionic accumulation (ABOOD and ROMANCHEK, 1955), the suggestion offered itself that mucopolysaccharides may play a role, since they are implicated in ionic transfer elsewhere in the organism. The present communication reports the histochemical identification of hyaluronic acid (HA)‡ in nerve, its isolation and chemical characterization, as well as certain cationic-exchange studies pointing to its possible role in nerve conduction.

METHODS

Preparation of hyaluronic acid. Sciatic and brachial nerves from 10 bullfrogs (net weight about 8 g) were stripped of their connective tissue and placed in 50 ml of a mixture of equal volumes of acetone and ether at 25° for 1 hr. The nerves were thoroughly homogenized in 20 volumes of 0·02 N sodium hydroxide and the homogenate mechanically stirred for 30 min. Upon the addition of 2 volumes of ice-cold acetone, a stringy amorphous precipitate formed which floated to the surface and had a tendency to adhere to a glass stirring rod. The amorphous agglomeration was then homogenized in water and reprecipitated in 2 volumes of acetone. After centrifugation at 0°C, the residue was homogenized thoroughly in 35 ml of 1 M sodium acetate until the agglomeration was completely disintegrated. Sedimentation of the homogenate at 20,000 × g in the Spinco ultracentrifuge yielded a slightly milky supernatant which formed a white amorphous precipitate when poured into 2 volumes of an ice-cold 3 : 1 (v/v) alcohol-acetone mixture. Further purification could be achieved by re-homogenizing the material in sodium acetate and repeating the subsequent procedure. The final material was resuspended in 0·2 M sodium acetate, dialysed against distilled water at 0°

* This research was supported by a contract between the Office of Naval Research and the University of Illinois.

† Present address, Department of Pathology, University of California School of Medicine.

‡ The abbreviation HA was used for hyaluronic acid, whether or not the material was "pure" or crudely purified (i.e., containing protein). Except as otherwise indicated, the material referred to as "purified" in the text has the composition as given in Table 1 under "2nd alcohol ppt."

for 6 h, and lyophilized. In an effort to remove part of the bound protein the nerve material was extracted with organic solvents. The material was suspended in 0·02 M sodium acetate and shaken in a separating funnel with 3 volumes of equal parts chloroform and amyl acetate. After the phases were separated by centrifugation in the cold, the aqueous layer was removed and the extraction procedure was repeated. This material was dialysed and lyophilized before analysis.

Protein. Protein content was determined from total nitrogen (NESSLER's reagent) and total amino acids (ninhydrin) after hydrolysis of the nerve material for 12 hr in 6 N HCl. Appropriate corrections were made for glucosamine. Paper chromatography of the hydrolysate was performed by the ascending technique, using an *n*-butanol-glacial acetic-H_2O mixture (40 : 10 : 50 by volume). A sample of serum albumin was treated in an identical manner and run simultaneously.

Hyaluronidase digestion. Ten mg of purified testicular hyaluronidase* (270 TR units/g), was incubated with 10 mg of the purified nerve mucopolysaccharide in 0·05 M acetate buffer at *p*H 5·5 in a final volume of 3·0 ml. The mixture was covered with toluene and incubated at 37°. Aliquots were removed at various times and analysed for reducing sugar and glucosamine. The hydrolysate to be chromatographed was treated with 1 N perchloric acid, the perchlorate removed from the supernatant as the potassium salt, after neutralization with 1 N potassium hydroxide, and the resulting supernatant concentrated and chromatographed (ascending) on Whatman No. 1 filter paper with butanol : acetic acid : water (40 : 10 : 50) as the developing solvent (PARTRIDGE, 1948). Identification of acetylglucosamine was done with dimethylaminobenzaldehyde according to PARTRIDGE (1948).

Glucosamine. Glucosamine was determined by a modification of the method of ELSON and MORGAN (1933). A sample of the nerve mucopolysaccharide was hydrolysed in 3 N HCl in a sealed vial for 3 hr. After the material was dried *in vacuo* over soda lime, it was redissolved in distilled water at *p*H 8·0. To 1·0 ml of the acid hydrolysate was added 1·0 ml of 1% w/v acetylacetone in *n*-butanol and 0·05 ml of 10 N KOH. After the solution was heated for 10 min in a boiling water bath, 1·0 ml of dimethylaminobenzaldehyde (200 mg in 5·0 ml of concentrated HCl + 15 ml ethanol brought to 50 ml with water) was added and the mixture placed in the boiling water bath for an additional 10 minutes. To the mixture was added 1 ml of ethanol and the optical density read 490 mμ.

Glucuronic acid. The method of DISCHE (1947) was used as described. A sample of pure HA was run simultaneously with the nerve material. The rate of development of the red-violet colour after carbazole addition, the absorption spectrum, and the rate of appearance of the deep violet colour after the addition of distilled water was identical for the two substances.

Histochemistry. The histochemical technique for the demonstration of unsulfonated (RINEHART and ABUL-HAJ, 1951) and sulphonated (ABUL-HAJ and RINEHART, 1952) mucopolysaccharides was used as previously described. Fresh frog and bovine sciatic nerves were fixed in 10 per cent formalin overnight, embedded in paraffin, and sectioned 5–10 μ thick. Alternate sections were incubated for 16 hr in a solution of hyaluronidase containing 150 TR units of activity/ml.

Ion-exchange studies. The material (about 30 mg dried material) to be used for cationic-adsorption studies was converted into the acid form by suspending it in 20 ml of 2 N acetic acid in 50% acetone for 20 min. After the centrifuged residue was washed twice in 50 ml of 50% acetone, it was homogenized in 20 ml of 0·02 N equimolar concentration of sodium and potassium hydroxide. To the homogenate was added 2 volumes of acetone, and the precipitate obtained was washed twice with 20 ml of 50% acetone. The material, which contained 10 micromoles of sodium and 5 micromoles of potassium, was then suspended in 5 ml of equal parts of 10^{-3} M potassium and sodium chloride, and dialysed (casein tubing) against 20 ml of this same salt solution for 24 hr at 5°. After the nerve HA was removed by centrifugation, potassium and sodium were determined on the external solution and on the solution within the dialysis bag by means of the flame-photometer attachment to the Beckman DU spectrophotometer.

Viscosity was measured with the Ostwald viscosimeter. Water absorption was determined by homogenizing the dried nerve material in 0·02 N sodium hydroxide and then centrifuging the suspension in an ordinary blood hematocrit tube. The increase in volume of the nerve HA was a measure of water absorption.

* The authors would like to thank Dr. H. J. ANDERSON of Cudahy Laboratories for his generous supply of hyaluronidase and hyaluronic acid; and the Research Division of Armour and Company for their co-operation in obtaining bovine sciatic nerves.

VOL 1 1956/

VOL.
1
56/57

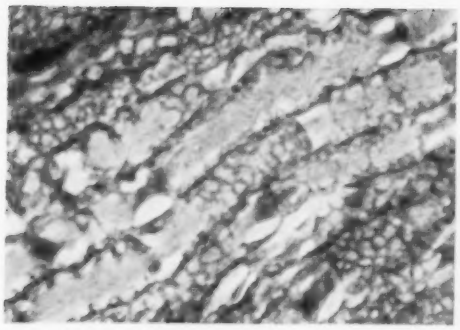


Fig. 1a Longitudinal section of frog sciatic nerve.

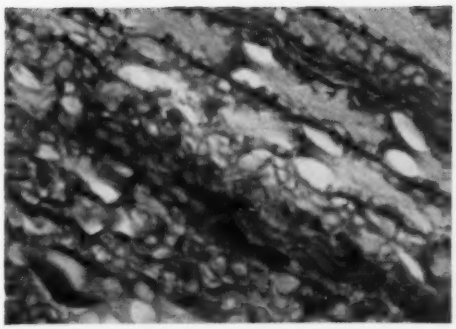


Fig. 1b Same as 1a, except pretreated with hyaluronidase.

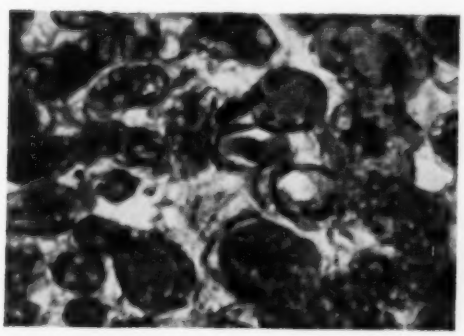


Fig. 2a Cross section of frog sciatic nerves stained for mucopolysaccharide (blue).

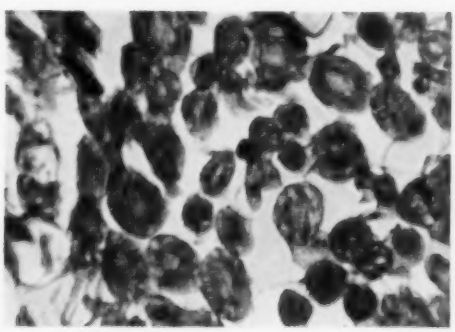


Fig. 2b Same as 2a, except pretreated with hyaluronidase.

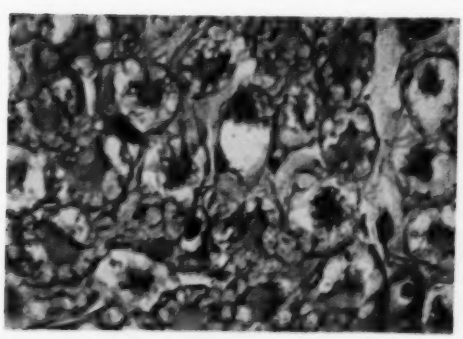


Fig. 3 Cross section of bovine sciatic nerve stained for mucopolysaccharide.

Fig. 4 Longitudinal section of bovine sciatic nerve.

VOL
1
1956/

RESULTS

Histochemistry. The presence of mucopolysaccharide could be histochemically demonstrated, both in the axoplasm and neurilemma sheath (Figs. 1a and 1b). In the axoplasm, the material appears as a diffuse mass (blue) throughout with a more deeply stained fibrillar structure imbedded within it. Around the nodes of Ranvier the material is especially dense. The neurilemma sheath stains even more intensely, particularly in the region of the Schwann cell. In cross-sections of the nerve (Figs. 2a and 2b), the myelin sheath appears as a porous envelope, staining orange-red, with frond-like processes extending at regular intervals from the mucopolysaccharide of the axoplasm into the sheath. In the longitudinal sections, these frond-like processes appear as a polygonal structure imparting a honeycomb effect. None of these structures showed a positive reaction for mucopolysaccharide (1b, 2b) when the sections were preincubated for 12 hours in testicular hyaluronidase (200 TR/ml frog's Ringer solution, pH 7·0). Sections of bovine sciatic nerve are presented in Figs. 3 and 4.

With the exception of a very thin (about 0·3 μ) layer between the nurilemmal and the myelin sheath, the histochemical reaction for sulphonation of the mucopolysaccharide in frog and bovine sciatic nerves was completely negative (plates not shown).

The composition of the purified HA from nerve is presented in Table 1. With repeated alcohol precipitation and chloroform-amylacetate extraction, the protein content of the material could be reduced from 60 to 32% by weight, while the glucosamine concentration rose from 10 to 19%, and the glucuronic acid from 9 to 21%. The chromatographic pattern of the protein hydrolysate resembled that of albumin, although preliminary studies with paper electrophoresis suggest the presence of at least one additional protein. Upon purification, the ash content (largely K and Na) increased from 12 to 16%, while the total nitrogen decreased but slightly. As determined by an Ostwald viscosimeter, the viscosity of the material improved with purification. The nerve material exhibited a great imbibition of water, absorbing about 400 times its volume (Table 1).

TABLE 1.—COMPOSITION OF THE MUCOPOLYSACCHARIDE FROM FROG SCIATIC NERVES. TOTAL N AND PROTEIN, AS DETERMINED BY NINHYDRIN, ARE CORRECTED FOR GLUCOSAMINE

	1st alcohol ppt. %	2nd alcohol ppt. %	chloroform-amylacetate extracted ppt. %
Ash	12	15	16
Total N minus glucosamine-N	11	8	8
Protein corrected for glucosamine	60	45	32
Glucosamine	10	13	19
Glucuronic acid	9	14	21
Viscosity	low	normal	normal
H_2O binding (ml H_2O/mg)	0·40	0·32	0·25

Hydrolysis of the nerve HA by testicular hyaluronidase resulted in a gradual increase of total reducing sugar (FOLIN and MALMROS, 1929) and acetylglucosamine (Fig. 5). The hydrolysis resulted in the formation of oligosaccharides, which on the basis of comparison with known HA from vitreous humour, appeared to consist of a tetrasaccharide (LINKER, MEYER, and HOFFMAN, 1956) and, to a slight extent, a larger

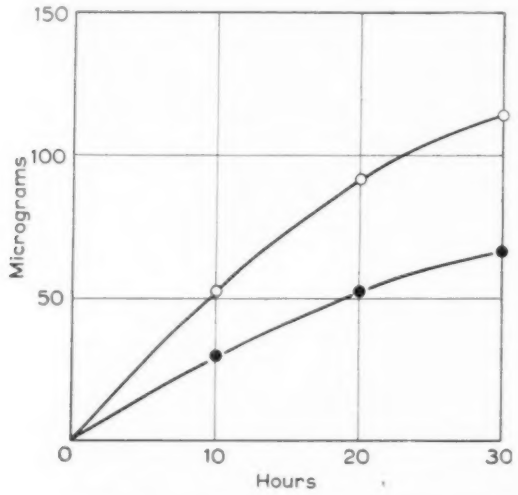


FIG. 5.—Increase in reducing sugar (○) and acetylglucosamine (●) on hydrolysis of nerve HA by testicular hyaluronidaes. Each point in the figure represents about 0·5 mg of the original nerve material.

oligosaccharide (Table 2). With EHRLICH's reagent the chromatographic spots gave a violet colour identical with acetylglucosamine in contradistinction to the orange-red of glucosamine. The chromatographic pattern of the nerve HA hydrolysate was almost identical with that of the vitreous-humour material.

TABLE 2.—IDENTIFICATION OF PAPER CHROMATOGRAPHIC SPOTS AFTER HA HYDROLYSIS OF NERVE AND KNOWN HA FROM BOVINE VITREOUS HUMOUR BY BOVINE TESTICULAR HYALURONIDASE

R_f	Known material	Nerve HA hydrolysate	Colour
0·13	*d*-glucosamine	0	orange-red
0·23	acetylglucosamine	+	violet
0·20	HA hydrolysate (tetrasaccharide)	+++	violet
0·00	HA hydrolysate (oligosaccharide)	+	violet

Although the nerve mucopolysaccharide absorbed about 3 times as much Na as K when homogenized in 0·02 N equimolar concentration of sodium and potassium hydroxide, it nonetheless exhibited some specificity for K accumulation at equilibrium

conditions during dialysis (Table 3). At equilibrium the concentration of K within the dialysis bag was 14% greater than in the surrounding medium in the case of pure Ha, and 47% and 41% greater in the case of the material of frog nerve and bovine nerve, respectively. It should be pointed out that the material exhibited no selectivity for K unless it was in the swollen state during dialysis.

TABLE 3.—DISTRIBUTION OF Na AND K BETWEEN INSIDE AND OUTSIDE OF THE DIALYSIS BAG AT EQUILIBRIUM CONDITIONS. RESULTS ARE AN AVERAGE OF NUMBER OF EXPERIMENTS INDICATED, AGREEING WITHIN 3%. APPROXIMATELY 10 mg (DRY WEIGHT) OF THE PURIFIED MATERIAL WAS USED. SEE TEXT FOR DETAILS.

	Exp.		Outside mM	Inside mM	Diff. mM
HA	3	Na	0·28	0·29	3
		K	0·24	0·30	14
Frog nerve HA	4	Na	0·28	0·30	7
		K	0·20	0·38	47
Bovine nerve HA	4	Na	0·28	0·30	7
		K	0·22	0·37	41

DISCUSSION

Both the chemical and physical characteristics of the nerve mucopolysaccharide suggest that the substance is a nonsulphonated HA resembling in most respects the classical material from vitreous humour or the umbilical cord. The chemical composition of our most highly purified nerve material agrees closely with the analytical data of OGSTON and STAINER (1950) for HA from synovial fluid. Their best preparations contained about 30% protein, which appeared to be albumin. Chromatographically, the amino acid pattern of protein hydrolysates occurring in association with HA of the nerve was also indistinguishable from crystalline bovine serum albumin. The nerve polysaccharide consists of equimolar concentrations of glucuronic acid and acetylglucosamine comparable to HA of umbilical cord (LINKER, MEYER, and WEISSMAN, 1955). In its physical characteristics, the nerve substance also resembles known HA: its sponge-like consistency; the disappearance of this amorphous property in solutions containing acids or electrolytes; its excessive imbibition of water, and positive double refraction of flow (OGSTON and STAINER, 1950). Furthermore, the chromatographic pattern of the enzymatic hydrolysate of the nerve material resembled that of known HA, which is presumably broken down to tetrasaccharides and a higher unknown oligosaccharide by testicular hyaluronidase (LINKER *et al.*, 1956).

HA is distributed in a comparatively high concentration in areas of the nerve fibre important to conduction, such as in the axoplasm (particularly at the nodes of Ranvier) and between the myelin and neurilemma sheath. Of particular significance is the fact that the total structural organization (fibrillar) and most of the contents of the axoplasm disappear upon treatment of the nerve sections with hyaluronidase. It is likely that the sol-like consistency of the axoplasm is, in part at least, attributable

to the presence of HA. Although the hyaluronic acid extracted from nerve may to some extent come from endoneurial and perineurial connective tissue, the histochemical preparations would indicate that the main source of the material is the axoplasm.

In view of the fact that mucopolysaccharides can act like a cation-exchange resin MEYER and RAPPORT (1951) and have a great affinity for alkali metals such as K and Na, it is tempting to speculate that the material may be of importance in ion transport and nerve conduction. The regulation of K and Na excretion by the kidney tubules, as well as fluid balance, may be associated with the presence of mucopolysaccharides. The Na retention, K diuresis, and generalized "interstitial oedema" occurring in the tissues as a result of desoxycorticosterone administration to unilaterally nephrectomized rats are accompanied by generalized swelling of mucopolysaccharides (RINEHART, 1955). Upon the administration of oestrogen to monkeys, the tremendous increase in the mucopolysaccharides which occurs in the sex skin is accompanied by an equally large accumulation of Na, K, and bound water (RINEHART and ABUL-HAJ, 1951; DURAN-REYNALS, 1950). All these findings leave little doubt of the importance of mucopolysaccharides in ion binding, and its presence in such large amounts in the axoplasm and neurilemma sheath is especially significant. There does appear to be some selectivity of the nerve HA for the smaller hydrated cations, such as K, and Rb, but this selectivity is small compared to the intracellular K accumulation in nerve. It is more likely that a highly structuralized complex of protein and HA in the intact nerve is involved in ionic accumulation, and that ionic transport during activity is the consequence of structural alteration within the complex. The failure of the nerve material to selectively absorb K except in the hydrated, expanded state indicates that a certain structural configuration of the HA-protein complex is involved in selectivity, a concept in conformity with the "fixed charge hypothesis" of LING (1952).

Optical and mechanical changes have been observed in axons during excitation (BRYANT and TOBIAS, 1955), and to what extent this can be ascribed to changes in the axoplasmic HA-protein complex remains to be determined. It is conceivable that since HA is a polysaccharide with a molecular weight of 200,000, a reversible process of depolymerization and repolymerization could accompany activity, and, thereby, influence ionic transport.* Future work along this general line is contemplated in our laboratory.

HESS (1955a) has studied the appearance of the ground substance in the developing central nervous system, using histochemical techniques. He suggests that in addition to providing the colloidal substrate for the orientation of the developing fibres of the central nervous system (HESS, 1955a), mucopolysaccharides may be a contributory factor to the blood-brain barrier (HESS, 1955b).

Preliminary attempts to determine the effect of testicular hyaluronidase on the electrical properties of nerve have yielded largely negative results, although a slight decrease in conduction time was recorded. The frog sciatic nerve bundle was merely soaked in the enzyme solution, a procedure which left the axoplasmic HA unchanged.

* SHURRAGER (1956) has observed many interesting changes in the hyaluronic acid of vitreous humor during light and dark adaptation, which are believed to be the result of depolymerization and repolymerization of HA. These changes are extremely rapid, occurring in a matter of a few seconds. In addition to its extreme sensitivity to light, the vitreous humor material is responsive to changes in temperature. SHURRAGER has also indicated that similar changes are occurring in the cerebral cortex during electrical excitation.

VOL
1
1956/

NORDQUIST (1952) was able to show that hyaluronidase alters the permeability of the connective tissue of frog nerve, but had no effect otherwise. TOBIAS (1955) was also unable to observe any effect of the enzyme on nerve conduction. It is unlikely that the hyaluronidase was able to penetrate the myelin sheath in such studies. Experiments are now under way in our laboratory to determine the effect of microinjection of the enzyme into the axoplasm, using, in particular, bacterial hyaluronidases which split HA to the disaccharide unit (LINKER *et al.*, 1956).

SUMMARY

The presence of unsulphonated hyaluronic acid has been histochemically demonstrated in the axoplasm and neurilemma sheath of amphibian and mammalian nerve. The material has been partially purified from frog sciatic nerve; and its similarity to known hyaluronic acid established by chemical and enzymatic means. Since hyaluronic acid acts like a cationic exchange resin and does seem to bind potassium in preference to sodium, the suggestion is offered that hyaluronic acid may have a significant role in nerve conduction.

REFERENCES

ABOOD L. G. and GOLDMAN E. (1956) *Amer. J. Physiol.* **184,** 329.
ABOOD L. G. and ROMANCHEK L. (1955) *Exp. Cell. Res.* **8,** 459.
ABUL-HAJ S. K. and RINEHART J. F. (1952) *J. Nat. Cancer Inst.* **13,** 232.
BRYANT S. H. and TOBIAS J. M. (1955) *J. Cell. Comp. Physiol.* **46,** 71.
DISCHE Z. (1947) *J. Biol. Chem.* **167,** 191.
DURAN-REYNALS F. (1950) *Ann. N.Y. Acad. Sci.* **52,** 946.
ELSON L. A. and MORGAN W. T. J. (1933) *Biochem. J.* **27,** 1824.
FOLCH J. (1952) in *Biology of Mental Health and Disease*, P. Hoeber, N.Y.
FOLIN O. and MALMROS H. (1929) *J. Biol. Chem.* **83,** 115.
HESS A. (1955a) *J. Comp. Neurol.* **102,** 65.
HESS A. (1955b) *Arch. Neurol. and Psychiat.* **73,** 380.
JOSEPH N. R., ENGEL M. B, and CATCHPOLE H. R. (1952) *Biochim. et biophys. Acta* **8,** 575.
LING G. (1955) *Amer. J. Phys. Med.* **34,** 89.
LING G. N. (1952) in *Phosphorus Metabolism* II, 748, Johns Hopkins.
LINKER A., MEYER K., and HOFFMAN P. (1956) *J. Biol. Chem.* **219,** 13.
LINKER A., MEYER K., and WEISSMAN B. (1955) *J. Biol. Chem.* **213,** 237.
MEYER K. and A., and RAPPORT M. M. (1951) *Science* **113,** 596.
NORDQUIST P. (1952) *Acta pharmacol. et toxicol* **8,** 195.
OGSTON A. G. and STAINER J. E. (1950) *Biochem. J.* **46,** 364.
PARTRIDGE S. M. (1948) *Biochem. J.* **42,** 238.
RINEHART J. F. (1955) *Ann. N.Y. Acad. Sci.* **61,** 684.
RINEHART J. F. and ABUL-HAJ S. K. (1951) *Arch. Path.* **52,** 189.
SHURRAGER P. S. (1956) Personal communication.
STEINBACH H. B. (1950) *Transactions on First Conference on Nerve Impulse* Josiah Macy Jr. Found., New York.
TOBIAS J. M. (1955) *J. Cell. Comp. Physiol.* **46,** 183.

Journal of Neurochemistry, 1956, Vol. 1 ,pp. 126–133, Pergamon Press Ltd., London.

THE CHANGES WITH AGE IN THE PROTEIN COMPOSITION OF THE RAT BRAIN

DORIS H. CLOUET and M. K. GAITONDE
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(*Received* 25 *May* 1956)

VOL
1
1956/

THE proportion of protein in the rat brain on a dry-weight basis remains relatively constant during the period of foetal and subsequent growth when the brain is most rapidly increasing in size. It is not known whether the chemical composition of the protein remains approximately constant during growth, or whether the ratios of the individual amino acids in the proteins vary significantly with the age of the animal. However, such parameters as enzymic activity and nucleoprotein content show that differentiation of the protein must occur to some extent. In this connexion a report of BLOCK (1937b) that the concentration of the basic amino acids arginine and histidine is lower in young than in adult rat brain is of interest. BLOCK's data suggest also that owing to a relatively lower concentration of histidine in young animals, the arginine-histidine ratio (A/H) is increased.

In the present investigation a study was made of the protein composition of the rat brain in relation to the age of the animal. The arginine and histidine content of the proteins were determined in the brain of animals of different ages, and in particular regions of the brain representing different stages of ontogenetic development. Simultaneous determinations in muscle were carried out to aid in recognizing general growth phenomena. Measurements of the body weight, brain weight, and water content of the brain in rats of different ages were obtained to provide reference standards for these experiments.

EXPERIMENTAL

Albino rats of the Wistar strain were used throughout this experiment. For each age group four rats were examined; of these not more than two were from any one litter. Littermates were used as far as possible to provide a comparison in other age groups. To obtain sufficient material to get data for the foetal animals, entire litters with an approximate antepartum age taken from the time of mating were pooled. To obtain representive samples of particular regions of the brain, additional groups of from three to six rats were used at each age.

The rats were decapitated and their brains, including the cerebellum and medulla, were removed. The brains were weighed and homogenized in 10 ml of 10% (w/v) trichloroacetic acid. Those to be dissected were kept cold on the upper surface of a Petri dish filled with ice-salt mixture. From the brains were dissected samples of

(a) the frontal cortex, (b) midbrain, (c) medulla, and (d) the intact olfactory bulbs, (e) hypothalamus, and (f) cerebellum. The pooled samples of each region were weighed and homogenized in 10 ml of 10% trichloroacetic acid. These suspensions were centrifuged, and the lipids removed from the precipitate by successive extractions with 30 ml each of cold acetone, chloroform: ethanol (1 : 1), and ether. The weight of the protein fraction was obtained after drying it at 120° for 30–45 min. The weight of the lipid fraction was obtained after evaporating the organic solvents from the combined extract. The protein fraction separated under these conditions contained a proportion of lipoprotein stable to treatment with TCA and extraction with cold lipid solvents. At each age dry weights were obtained in separate experiments by heating the whole brain or the dissected region in an oven at 120° to a constant weight. A sample of the gastronemius muscle from each rat in the age series was treated in the same manner.

The protein was hydrolysed by adding 5 ml of 6N HCl to the residue, which was then kept in sealed tubes at 120° overnight.

The hydrolysate was filtered through a Whatman No. 40 filter paper, neutralized, and diluted with water. Arginine was determined on an aliquot by a modified Sakaguchi method (CLOUET, GAITONDE, and RICHTER, 1956). Nitrogen was determined on a sample by a micro-Kjeldahl analysis.

Arginine could be estimated on the whole hydrolysate of tissue proteins, but histidine analyses were affected by contaminating substances in the hydrolysate. Therefore, a preliminary adsorption on charcoal was necessary before histidine was measured. To a sample of the hydrolysate containing about 1·5 mg of dry protein was added 100 mg of acid-washed charcoal and enough HCl to make the solution 1N with respect to this acid. After centrifugation, the charcoal was washed four times with 2 ml of 1N HCl at 60–70°. The acid washings were added to the original filtrate from the charcoal adsorption. This solution was neutralized with 6N NaOH and diluted to 20 ml with water. Histidine was determined in this solution by a modification of the Pauly colorimetric method (CLOUET, GAITONDE, and RICHTER, 1956).

In the charcoal adsorption procedure recoveries of 95–98% of added histidine were obtained. Tyrosine and histamine did not interfere. Arginine was recovered from the charcoal under these conditions only to the extent of 65–70%, and therefore was estimated in the original hydrolysate.

Protein values were calculated by multiplying the nitrogen value by the average factor in each age group (6·78–6·94 for young rats and 7·14–7·19 for the older animals).

RESULTS

In Table 1 the average body weight and brain weight as well as the nitrogen, protein, and lipid content of the brain are shown. In Fig. 1 the protein content of the brains of rats of various ages is expressed in terms of the total solids in the brain.

In Table 2, the nitrogen content of the protein in six regions of the brain is shown for six age groups. The medulla alone of the regions measured contained a protein with a higher nitrogen content ($P = 0{\cdot}01$) in both young and older rats. All of the other regions did not differ from the mean significantly. The average amount of nitrogen was generally higher in the brains of the younger group of animals.

Table 3 shows the protein content of each area of the brain on a wet weight basis.

The relative positions of the various areas may be seen to be: medulla > hypothalamus > cerebellum > midbrain > olfactory bulb > neocortex for the young animals and midbrain > neocortex > cerebellum > hypothalamus > olfactory bulb > medulla for the older animals.

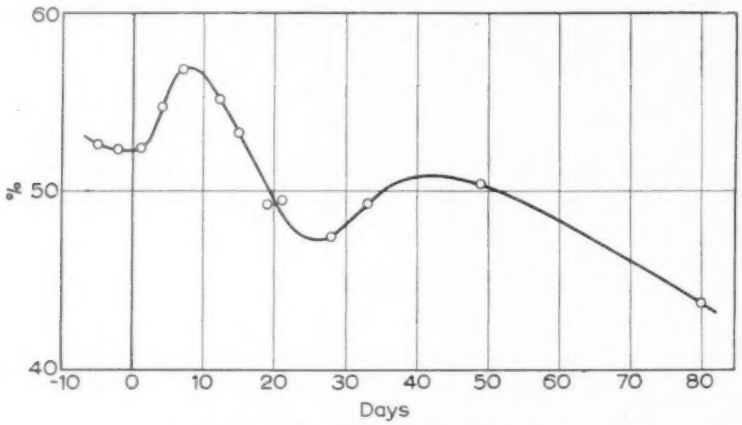


FIG. 1.—Proportion of proteins in brain solids.
The proportion of protein in the total solids of rat brain is shown in rats of various ages. Each point represents at least four separate animals and analyses.

TABLE 1.—PROTEIN CONTENT OF RAT BRAIN

Age days	Number of rats	Body weight (g)	Brain weight (g)	Solids % wet weight	Brain nitrogen (mg)	Protein % wet weight	Lipid % wet weight
5 ante-partum	6	1·73	0·087	14·65	0·76	7·72	6·98
2 ante-partum	10	3·00	0·130	12·54	1·32	6·58	5·97
1	8	5·58	0·204	12·36	1·96	6·52	5·98
4	4	5·58	0·267	11·20	2·41	6·12	5·07
7	4	9·98	0·592	11·80	5·78	6·64	5·00
12	4	14·2	0·830	12·28	8·28	6·76	5·41
15	4	16·3	0·938	15·78	11·58	8·39	7·41
19	4	19·8	1·019	17·06	12·59	8·40	8·68
21	4	27·8	1·080	18·55	14·61	9·18	9·42
28	4	25·6	1·130	21·40	15·60	9·37	11·73
33	4	31·3	1·158	21·35	17·82	10·47	10·75
49	4	43·8	1·130	21·50	17·54	11·08	11·07
80	4	77·3	1·290	22·15	16·67	9·21	12·00
245	4	329·5	1·470	22·54	21·38	10·38	12·37

Table 4 shows the amounts of arginine and histidine and the A/H ratio in the brain and muscle proteins. Table 5 shows the significance of these values when the rats were divided into young (1–40 days) and older (over 40 days) age groups. The mean

TABLE 2.—NITROGEN CONTENT OF BRAIN PROTEINS
(g N/100 g protein)

Age days	Number	Frontal cortex	Olfactory bulb	Cerebellum	Medulla	Midbrain	Hypothalamus
7–17	11	14·1	15·0	13·6	16·5	13·7	13·5
52–84	11	13·6	14·5	13·9	15·8	12·4	13·5
Average		13·8	14·7	13·7	16·2	13·1	13·5

A test of the probability (P) of the differences in the nitrogen content being significant showed that only the nitrogen in the medulla proteins varied significantly. In the 7–17 day age groups $P = 0{\cdot}02$, in the 52–84 day age group $P = 0{\cdot}01$, and the average differed from the average nitrogen values ($P = 0{\cdot}01$).

TABLE 3.—PROTEIN CONTENT OF AREAS OF RAT BRAIN
(% of wet weight)

Age days	Number	Frontal cortex	Olfactory bulb	Cerebellum	Medulla	Midbrain	Hypothalamus
7	5	5·48	6·34	6·51	7·86	5·76	7·31
8	4	7·42	6·93	7·39	7·68	7·69	7·08
17	2	9·13	8·40	9·89	7·64	8·75	9·44
Average		6·85	6·93	7·44	7·75	7·01	7·59
52	3	10·35	9·25	10·82	7·13	11·75	10·23
75	2	9·56	7·72	10·26	8·50	10·68	8·90
58–84	6	12·75	9·92	12·01	9·08	12·42	9·54
Average		11·51	9·34	11·37	8·44	11·92	9·61

difference of the amounts of arginine and histidine in each age group was compared with the standard error of difference (s.e.d.) calculated according to CHAMBERS (1948). The means were considered significantly different if the arithmetic difference of means was at least twice the s.e.d. In the brain, the levels of arginine and histidine in the proteins of the older rats were significantly higher. In muscle, neither the histidine nor arginine levels were different. Parenthetically, it may be stated that when the protein values were calculated on the basis of the customary factor of 6·25 times the nitrogen content, the arginine and histidine showed the same significant differences.

In Table 6, are shown the arginine and histidine concentrations in six regions of brain in two groups of eleven rats each, again divided at the 40th day. A/H ratios for each brain area are also given here. When the data were treated statistically, there was greater diversity in the A/H ratio in the various areas of the brains of the older rats when compared to those in the younger group ($P = 0{\cdot}13$). As before there were lower arginine and histidine concentrations in the proteins of the brains of the younger rats and lower A/H ratios in the older animals.

TABLE 4.—ARGININE AND HISTIDINE IN BRAIN AND MUSCLE PROTEIN
(g /100 g protein)

Age	Nos.	Brain			Muscle		
		Arginine	Histidine	A/H	Arginine	Histidine	A/H
5 days ante-partum	6	11·64 ± 0·60*	3·60 ± 0·25	3·24	5·35†	2·10†	2·55
2 days ante-partum	10	— —	2·67 ± 0·26	—	—	2·50†	—
1st week	12	7·87 ± 0·94	2·43 ± 0·24	3·24	7·12 ± 0·92	2·55 ± 0·34	2·80
2nd week	8	7·06 ± 0·50	2·64 ± 0·31	2·67	6·96 ± 0·59	2·03 ± 0·24	3·43
3rd week	8	7·77 ± 0·88	2·69 ± 0·21	2·88	6·05 ± 0·98	2·68 ± 0·93	3·50
4th week	8	8·32 ± 0·77	3·19 ± 0·28	2·62	5·97 ± 0·48	2·68 ± 0·93	2·24
5th week	4	8·41 ± 0·26	3·28 ± 0·13	2·57	6·64 ± 0·30	2·68 ± 0·17	2·48
7th week	4	8·84 ± 0·62	3·47 ± 0·63	2·54	5·30 ± 0·68	1·76 ± 0·12	3·01
8th week	6	8·96†	3·71†	2·42	—	—	—
12th week	4	9·28 ± 0·65	3·87 ± 0·23	2·39	6·55 ± 0·18	2·39 ± 0·36	2·74
35th week	4	8·99 ± 0·47	3·80 ± 0·14	2·37	6·27 ± 0·24	2·28 ± 0·24	2·75

A/H = Arginine : histidine ratio.
* Standard deviation.
† Single value of pooled sample.

TABLE 5.—SIGNIFICANCE OF DIFFERENCES IN AMOUNT OF ARGININE AND HISTIDINE IN BRAIN AND MUSCLE PROTEIN

	(g/100 g protein)			S.e.d.*	*P*†
	Mean 1–40 days	Mean over 40 days	Difference		
Brain					
Arginine	7·86	8·89	1·03	0·30	<0·01
Histidine	2·77	3·71	0·94	0·17	0·001
A/H	2·84	2·39			
Muscle					
Arginine	6·55	6·04	0·51	0·34	0·2
Histidine	2·33	2·14	0·19	0·19	0·4
A/H	2·81	2·82			

* S.e.d. = standard error of difference.
† *P* = probability calculated on the basis of the *t* value in small samples.

VOL 1 1956/

TABLE 6.—ARGININE AND HISTIDINE IN PROTEINS OF DIFFERENT REGIONS OF THE BRAIN (g/100 g protein)

Age	Frontal cortex	Olfactory bulbs	Cerebellum	Medulla	Midbrain	Hypothal-amus
7–17 *days* (11 rats)						
arginine	6·82	6·80	7·22	6·66	6·82	6·92
histidine	2·53	2·71	2·68	2·47	2·74	2·88
A/H	2·69	2·51	2·69	2·67	2·49	2·41
52–84 days (11 rats)						
arginine	7·61	8·37	8·49	7·91	8·10	8·12
histidine	3·41	3·68	3·24	3·19	3·60	3·95
A/H	2·23	2·28	2·62	2·48	2·25	2·06

DISCUSSION

It is clear from the data given in Table 1 that the nitrogen and protein content of the rat brain depended on the age of the animal. The absolute content of nitrogen, and consequently protein, in the brain showed a regular increase with age. Figures for the foetal brain were very close to those of one-day-old rats. However, when the protein content was related to the total solids in brain (Fig. 1.) it showed a peak at the seventh day. When the protein concentration was calculated on a wet-weight basis, this peak was masked by the relatively greater water content of the brains of the younger animals.

The average amount of nitrogen in the protein fractions of the brains of rats from 1–33 days old was 14·74% and in rats over 40 days, 14·01%. The corresponding figures for protein from the six regions of brain were quite similar: 14·45% and 13·95%. These values are low when compared with 16% which has been found as an average in most proteins, but they may be expected from a consideration of the high content of lipoprotein in the brain.

KOCH and KOCH (1913) studied the lipids and proteins of mixed cerebral tissue from rats of six ages from one to 210 days of age. This early work is extended, particularly in the ages of 1–23 days, by the data in Table 1.

From Table 2 it can be seen that the protein of each region appears to have a characteristic nitrogen content which is not greatly affected by age.

The values obtained for the protein content of the different regions of the rat brain give additional evidence for the view that the phylogenetically older parts develop first, and are superseded by the newer parts as the animal matures. The vascular bed of the different regions shows approximately the same order of maturation (CRAIGIE, 1924, 1925, 1931) and this may be presumed to be a necessary corollary either preceding or succeeding the increase in protein. The respiration rate of rat brain tissue *in vitro*, which was described by TYLER and VAN HARREVELD (1942), is related to the amount of protein in the brain parts, as is the acetylcholine esterase activity (ELKES and TODRICK, 1955). However the enzyme activity is not necessarily

related to the total protein concentration: the level of certain respiratory enzymes was found to increase disproportionately to the total protein content of rat brain (POTTER *et al.*, 1945).

The variation in the arginine and histidine content of the brain proteins with the age of the animal was significant both when the change in the amino acids was related to the nitrogen content of the protein fraction, and when it was related to the total protein. The histidine level in the brain proteins fell after birth, regained its foetal level in about 20 days, then slowly increased, reaching a constant level about one-third higher at 80 days. The arginine level showed a similar pattern, although the decrease in the second week after birth was more pronounced and the increase from that value to the adult level was not relatively as great as that of histidine. Therefore the A/H ratio steadily decreased with the increasing size of the brain.

The choice of a dividing age between young and old rats must depend on the criteria one uses. The most rapid growth of the brain occurs in the rat from birth to the tenth day, when there is a fourfold increase in the brain weight. From the tenth to the twentieth day the rate of lipid deposition exceeds that of protein as myelinization begins. As myelinization is fairly complete after 40 days, a basis is provided for the choice of 40 days as a dividing line for the statistical study. From Table 4 it is apparent that the difference between the young and older rats would be greater if the animals were grouped according to an earlier dividing age. In the interpretation of these differences in arginine and histidine in proteins, such factors as the changing cell population of brain tissue with maturation, and the great increase in total protein during the first ten days, must be considered.

The data for the foetal brain were too few to draw conclusions about changes during foetal development. FLEXNER (1940) found that in the guinea pig there was no change during gestation in the amount of protein when this was related to the total weight of cortical cells. BLOCK's observation of a lower level of histidine in the proteins of 21-day-old rat as compared with adult animals was amply confirmed. A/H ratios have not been used previously to express change in the protein composition. However, these ratios may be calculated from the data in the literature (BLOCK and BOLLING, 1951). Young rats had an A/H ratio of 2·81, and adults 2·54. The ratio ranged from 2·09 to 3·04 in sheep, monkey and man without any reference to age or sex differences. An especially interesting observation may be made from the data given by BLOCK for humans (1937a). The A/H ratio in the brain protein of a patient suffering from late amaurotic idiocy was 4·06 compared with 2·54 for six nonpsychotic control adults.

The study of the muscle proteins was included to determine whether the change with age in A/H ratios was a general phenomenon or limited to the brain. The concentration of both arginine and histidine in muscle was lowest on the seventh week after birth. The A/H ratios showed no regular trend in contrast to those in the brain.

In the different parts of the brains of young rats the arginine and histidine content of the protein fraction was remarkably constant. The A/H ratios in both age groups were also constant from region to region. An apparent trend toward a phyletic order could be seen in the histidine content of different regions in the older animals.

VOL
1
1956/

SUMMARY

1. The protein content of the brain of rats of various ages from foetus to adult has been measured. A regular increase with age of the protein on a wet-weight basis was generally observed. When the protein was related to total solids, a peak of protein content occurred at about the 7th day postpartum.

The protein content of six regions of brain was also related to the age of the rat. The protein content of the brain of young rats decreased in the order: medulla > hypothalamus > cerebellum > midbrain > olfactory bulb > cortex. In the older rats the order was: midbrain > cortex > cerebellum > hypothalamus > olfactory bulb > medulla.

2. The concentrations of the amino acids, arginine and histidine, were significantly lower in the brain proteins of younger rats. The A/H ratio showed a progressive decrease from birth to adult age, due to a relatively greater increase in concentration of histidine.

The level of these two amino acids in the proteins of six regions of brain were determined.

Acknowledgements—The authors wish to thank Dr. DEREK RICHTER for his interest and advice during the course of this work. They also wish to thank the United States Public Health Service for a fellowship to D.H.C. and the Rockefeller Foundation for a grant to M.K.G.

REFERENCES

BLOCK R. J. (1937a) *J. Biol. Chem.* **120,** 467.
BLOCK R. J. (1937b) *J. Biol. Chem.* **120,** 769.
BLOCK R. J. and BOLLING D. (1951) *The amino acid composition of proteins and foods*, Charles C. Thomas, Springfield, Ill.
CHAMBERS E. G. (1948) *Statistical calculation for beginners*, Cambridge University Press.
CLOUET D. H., GAITONDE M. K., and RICHTER D. (1956) *J. Neurochem*, **1,** in the press.
CRAIGIE E. H. (1924–25) *J. Comp. Neurol.* **38,** 27.
CRAIGIE E. H. (1925) *J. Comp. Neurol.* **39,** 301.
CRAIGIE E. H. (1931) *J. Comp. Neurol.* **52,** 353.
ELKES J. and TODRICK A. (1955) *Biochemistry of the developing nervous system*, edited by H. WAELSCH, Academic Press, N.Y.
FLEXNER J. B. and FLEXNER L. B. (1950) *Anat. Rec.* **106,** 422.
KOCH W. and KOCH M. L. (1913) *J. Biol. Chem.* **15,** 423.
POTTER V. R., SCHNEIDER W. C., and LIEBL G. J. (1945) *Cancer Res.* **5,** 21.
TYLER D. B. and VAN HARREVELD A. (1942) *Amer. J. Physiol.* **136,** 600.

VOL. 1 56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 134 – 144. Pergamon Press Ltd., London

THE APPLICATION OF A SCANNING AND COMPUTING CELL ANALYSER TO NEUROCYTOLOGICAL PROBLEMS

HOLGER HYDÉN and SUNE LARSSON
Department of Histology, Faculty of Medicine, the University of Göteborg, Sweden

(*Received* 18 *June* 1956)

VOL
1
1956/

IT is now possible to apply quantitative cytochemical procedures, with the sources of error kept under control, for the analysis of biologically important substances in nerve cells and nervous tissue structures. Thus an ultramicrochemical method has been developed for the determination of ribonucleic acid (RNA) and the nucleotides of individual nerve cells (EDSTRÖM, 1953a, b; 1954). X-ray microradiography using soft X-rays is suitable for the study of the amount of dry substance which can be further divided into chemical fractions if fresh specimens are used (BRATTGÅRD and HYDÉN, 1954; HYDÉN, 1955; HALLÉN, 1956). There are, however, a vast number of cellular units in a nerve centre which have to be studied for each specific problem if significant results are to be obtained. It therefore seems desirable to include among the technical aids a data-collecting and -handling apparatus.

The following were the requirements for the instrument described below: the apparatus should be able to perform many measurements on many cells in a short time, and give information both on the amount of substance per cell or cell section as well as on the distribution of substance within the cell. It should be possible to vary the wavelength range used for the analysis. The apparatus should also fulfil these demands when the sample is a tissue section.

The result may be obtained in the form of a picture in figures giving the transmission values, since the machine prints the values in the order in which they are scanned in the sample. The apparatus has been changed in some respects from the original (BOURGHARDT, HYDÉN, and NYQUIST, 1955) and has been further developed. The apparatus now yields one single transmission value for each cell, i.e. the integral of the individual values computed within the scanned area. So far we have mostly used the apparatus to evaluate X-radiograms of nervous tissue sections taken at 8–10 Å. For this purpose a reference system consisting of a step-wedge of a suitable material is always exposed together with the sample.

Including the one discussed in this paper, five different instruments have now been described for recording light transmission in histological sections. The image of the sample is scanned mechanically by a disc or a band provided with apertures or by moving the sample over an aperture. TOLLES (1955) discusses the construction of an apparatus for scanning the sample with an aperture which is approximately 20 μ^2 referred to the object plane and which can read the optical density of cells in an area of 600 mm^2 in 12 minutes or less. The instrument is intended to be used for the diagnosis of exfoliated normal or abnormal cells in the form of a smear. LOMAKKA (1955) has described a function transformer to be used in connection with CASPERSSON'S ultraviolet microspectrograph to get the integrated transmission value of

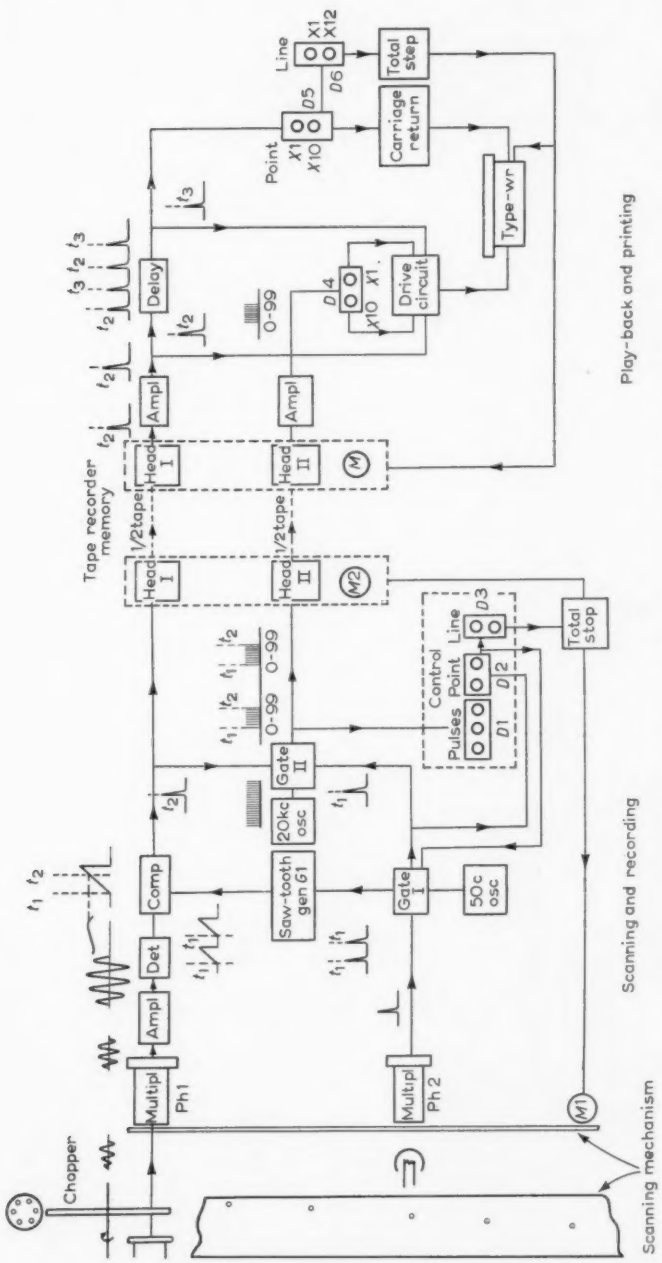


FIG. 1.—Block diagram of the apparatus. Cf. the text.

a track across the image of a cell. The apparatus transforms logarithmic values into linear and is coupled to an integrator. The advance of the line of scan is managed by hand. DEELEY (1955) has constructed an integrating microdensitometer for the rapid determination of the total absorption of cell sections. The image is scanned by a mechanically driven aperture, and a logarithmic circuit is used together with an integrator giving a meter reading linearly related to the total absorption. The image of one cell section is scanned in 3 seconds. THORNBURG (1956) has described a pulse-counting photometer for recording absorption spectra of tissue sections in visible as well as in ultra-violet regions.

DESCRIPTION AND PERFORMANCE

Our scanning and computing microphotometer consists of four parts: one microscope system with the scanning unit, one calculating unit, one magnetic storage unit, and one writing unit.

The stabilized light source consisting of a 100 W filament bulb can easily be substituted as can the optical system. Apochromats are used together with plane-compensating oculars. The scanning system consists of a 4-metre-long steel band, 60 mm in width and 0·051 mm in thickness, perforated by holes 0·3 mm in diameter. The holes are moved one diameter at a time, horizontally 100 diameters and vertically 120 lines. The resolving power of the scanning procedure is limited by the optics and other physical factors to an area of approximately 0·2 μ^2. In the case of film emulsions the limit seems to be 1 to 2 μ^2. The area of the sample scanned at the plane of the phototube is 30 × 36 mm, which area is thus divided into 12,000 measuring points.

The following general description of the apparatus refers to Fig. 1.

The radiation through the microscope system is modulated to 500 c/s by a rotating disc, and falls on the photocathode of the phototube. The alternating voltage emitted from the phototube is amplified and rectified and fed into a comparator circuit where it is compared with a saw-tooth voltage from a generator *G* 1. The saw-tooth voltage is started by the pulse t_1 and increases to the value of the rectified voltage from the detector which is proportional to the light intensity. At that instant a pulse t_2 is advanced to the gate II which now closes. This gate was opened at the moment t_1 by a pulse t_1 coming from gate I and advanced from a 50-c generator. To gate II pulses are advanced from a 20-kc oscillator. The conjoint action of the opening and closing pulse t_1 and t_2 respectively gives a pulse train the pulse number of which is determinated by the time $t_2 - t_1$.

In this way the radiation intensity from a point in the sample has been transformed to time. By the transformation to pulses the analogue process of the apparatus has been transferred into a digital decimal system.

The pulse train is fed into the head II of the tape recorder, one half band of which functions as a memory for the pulse train and the other half as a memory for the pulses t_2.

The pulse train is then transferred to a set of three dekatrons, *D* 1, which counts the number of pulses. The effector unit of the apparatus, the writer *W*, writes down the values in 200 figures per line. At the computation this procedure is regulated by gate I in the following way. An impulse from the phototube *Ph* 2, opens the gate, and the t_1 pulses from the 50-c oscillator are admitted and go also to a set of two dekatrons, *D* 2. When one hundred t_1 pulses have been counted by this unit a stop pulse is emitted which closes the gate I.

In this way is regulated the printing of 100 measuring values per line in the last stage of the process. The stop pulse goes also to another unit containing two dekatrons, *D* 3. When 120 pulses have been counted a pulse is advanced which stops the motors *M* 1 and *M* 2.

The time needed for the scanning and computing of 12,000 values amounts to approximately 4 minutes, i.e. a new value is measured fifty times per second. The writing unit, however, is not capable of writing it out on the paper at this speed, and therefore the typing occurs at one tenth of the speed.

The three dekatrons at *D* 1 can be substituted for another set containing seven dekatrons. If these are allowed to count the pulses continuously and are connected with a mechanical counter an average transmission value is obtained.

VOL
1
1956/

From head II the amplified pulse train is advanced to a dekatron unit, D 4, which counts the number of pulses. The tens digit of the dekatron is taken down by the amplified pulse t_2 from head I to the driving circuit of the writer, the coil of which strikes down the corresponding type. Since the typewriter cannot write two figures concurrently, a new pulse, called t_3, has been arranged. It is generated by the delay circuit, at a time instant which is between that of two t_2 pulses. The function of t_3 is to take down the units digit to the writer.

The pulse t_3 is also advanced to a dekatron unit, D 5, which emits an impulse to the circuit for the return of the carriage when the counting of one hundred t_3 has been completed. This impulse goes also to a further dekatron unit, D 6, which stops the whole apparatus when 120 pulses have been counted. In this way 100 transmission values per line for 120 lines have been computed, recorded, and finally scanned.

The numerical result of the scanning and computing procedure can also be obtained in another way, which may be of value for some biological investigations. As mentioned before, the dekatron unit at D 1 can be supplemented with four additional dekatrons. The set of seven dekatrons is connected to a mechanical counter. Thus a single value is obtained which is in fact the integral of all values within the scanned area. This possibility is useful to have when the distribution of substance within the cell can be neglected or when the sample is homogeneous from a structural point of view.

SOURCES OF ERROR

When photomultipliers are used to measure the light-intensity distribution over an area, the lack of uniformity of the photocathode can introduce an error and must therefore be eliminated.

The photocathode of the tube RCA 5819, which is used for this purpose, has been divided into 12,000 small areas (0·6 mm²). At even illumination the output from each of these has been registered. The variation in the sensitivity was found to be so large that it was considered hopeless to use the whole cathode surface for quantitative measurements. GODLOVE and WADEY (1954) have tested the same type of multiplier and found a sensitivity variation of the same magnitude. They divided the cathode surface in approximately 1000 areas.

In the construction of our scanner this error was eliminated by introducing a lens system in the light path by means of which the light from all scanned areas in the sample was focused to one single small spot in the centre of the photocathode within which the sensitivity was even.

RANDOM ERRORS

A variation of the mains voltage may introduce a small error in spite of the stabilization, as may small variations in the photomultiplier. These small errors, however, are compensated for when many measurements are carried out, e.g. ten measurements which give altogether 120,000 values.

The linearity of the output is dependent upon the linearity of the increase of the saw-tooth generator. This was proved by using a linearly increasing signal from a tone generator. The values printed by the machine were compared with those of the input signal measured as voltage. Fig. 2 shows the number of pulses as a function of the output of the tone generator.

The electronic part of the apparatus was tested by registering a value given by a tone generator, the output signal of which corresponds to that of the multiplier at an actual measurement (Fig. 3a). 2000 measurements were used and the standard deviation was found to be 0·494.

The performance of the electronic part plus the optical part was tested by recording 2000 transmission values from the same point in a sample (Fig. 3b). The

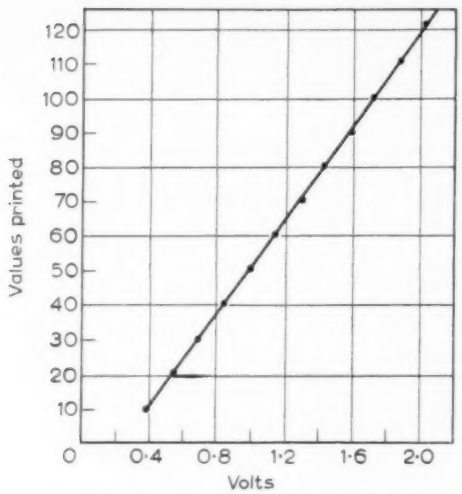


FIG. 2.—The linearity at the transformation of the analogue values to the decimal digital part of the apparatus.

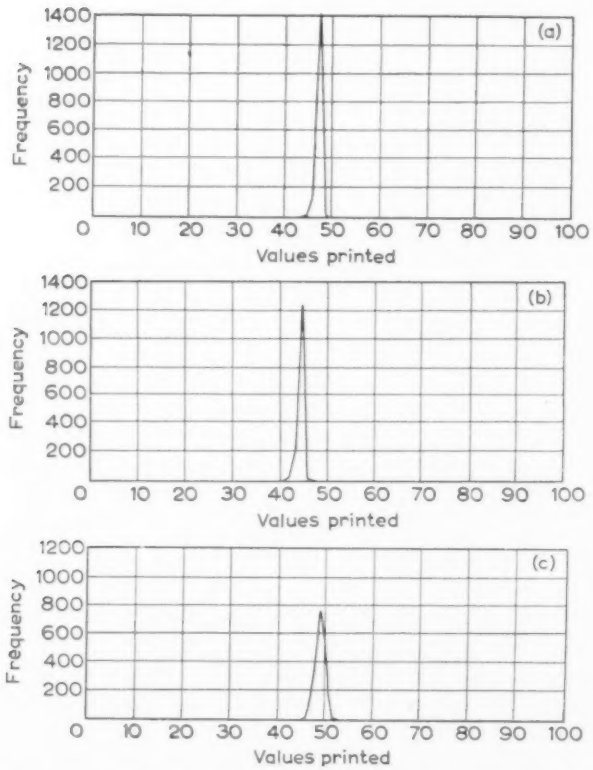


FIG. 3(a).—Frequency curve showing the dispersion of the values given by the electronic part.
FIG. 3(b).—The dispersion of the values given by the electronic plus the optical part at the calculation of one measuring point 2000 times.
FIG. 3(c).—The dispersion of the values when 2000 measuring points are scanned.

standard deviation was found to be 0·658; thus, errors arising from the optical part of the apparatus contribute a rather small fraction of the overall error.

DISTRIBUTIONAL EFFECT

The magnitude of the error of the whole apparatus, including the optical system, when the whole area of 30 × 36 mm is scanned, was calculated by using as a test object a step-wedge of thin nitrocellulose foil in 6 steps. This step-wedge was exposed to soft X-rays and the radiogram was used as a reference system for the transmission values 0 to 99. A uniformly absorbing surface with a transmission value approximately at steps 3–4 was scanned and computed by the apparatus. 2000 measurements were made, and the standard deviation was found to be 1·162. Fig. 3(c) shows a frequency curve. The error indicated in this figure, and deriving from spherical aberration in the microscope, thus amounts to 0·50 expressed as σ. In the actual measurements this additional error is easily compensated for by not using those values which fall in the periphery of the scanned image, or come from the parts of the optical system not satisfactorily corrected for spherical aberration.

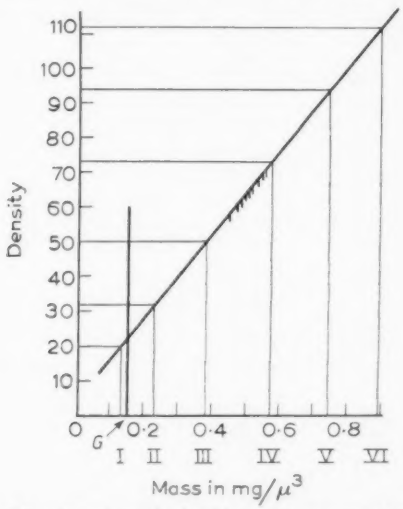


FIG. 4.—Diagram of density values obtained in an X-ray microradiogram. The mass values of the step-wedge (I–VI) are plotted against the density values obtained in a measurement of nerve cells (marked between steps III–IV).

APPLICATION TO NERVE CELLS

In biological problems involving the quantitative analysis of nerve cells, the following requirements often need to be considered. Firstly, it may be desired that many values should be obtained from many cells in a short time. Secondly, a very detailed analysis of a limited number of cells may be necessary. In the latter case the limit of the method's resolving power is usually approached. Examples are given below for both procedures in determining the amount of dry substance per unit volume.

The biological sections were placed on a supporting aluminium foil, 2 μ thick and sufficiently even, since it was produced by vaporizing metal. The sample was exposed at 8–10 Å on a fine-grained film emulsion together with a reference system with the same wavelength characteristics as the biological sample. It consisted of

nitrocellulose foil forming a step-wedge every step of which is approximately 1 μ thick. The thickness of the steps used was measured interferometrically. In the wavelength region used the X-ray absorption is proportional to the amount of C, N, and O in the sample, i.e. the main constituents of the organic cell material. The transmission of the structures in the radiogram was then evaluated by the scanning apparatus. The mass values of the reference system in mg per μ^3 are plotted against the density values obtained (see example in Fig. 4). The mass values of the cell structures are then obtained by transferring their density values to the mass values of the reference system.

FIXED NERVE CELL MATERIAL

Spinal ganglion cells, rabbit, fixed in CARNOY's solution.

Fig. 5(a) shows the section stained with gallocyanine after completed analysis and photographed in an Ultropak system (Leitz) at 300× magnification; 5(b) shows the same section exposed at 8–10 Å, and 5(c) the picture in density values of the same cells as in (a) and (b), calculated and printed by the scanning apparatus. When this square of figures has been obtained the X-radiogram is projected over it at a corresponding magnification, and the outlines of the cell structures are drawn by a pencil.

Multicell analysis. Sections through 130 spinal ganglion cells were analyzed at 100× magnification at the scanning plane. Optics: Zeiss apochromat 10× n.a. 0·30, Table 1. The cells were distributed on four sections and 70 measuring values on an average were obtained on every cell. An examination of the intracellular distribution of the values shows that the mass values of the nucleus, including the nucleolus, were approximately the same. This is due to the fact that the apparatus cannot discern between the transmission values of the nucleolus and the nucleus respectively at this magnification and with the optics used. The cytoplasm shows here and there an uneveness in the distribution of material depending, however, on fixation artefacts. The effect of the fixation is a shrinkage with a local concentration increase in peripheral cell parts.

TABLE 1

	Number of cells	Number of measurements	Mean of the mass in mg $10^{-9}/\mu^3$	σ	v%*
Cytoplasm	130	8961	0·23	0·04	18·3
Nucleus + nucleolus	36	417	0·21	0·05	23·8

* % denotes $\frac{\sigma \times 100}{\text{mean}}$

From the v value it is evident that the intercell variation is considerable, which is likewise reflected in the X-radiograms and which can be expected in fixed nervous tissue. An examination of the distribution of the mass values in fixed ganglion cell material shows gradients in the concentration which do not, however, as mentioned

VOL. 1 56/57

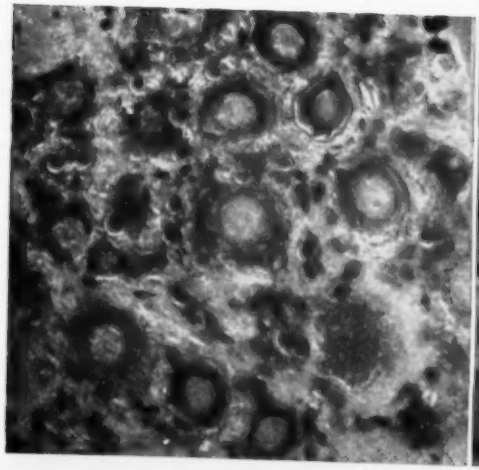


FIG. 5(a).—7-μ-thick section of a Carnoy fixed spinal ganglion, rabbit, stained with gallo-cyanine and photographed after the analysis in an Ultropak System (Leitz). Magnification. 3000×.

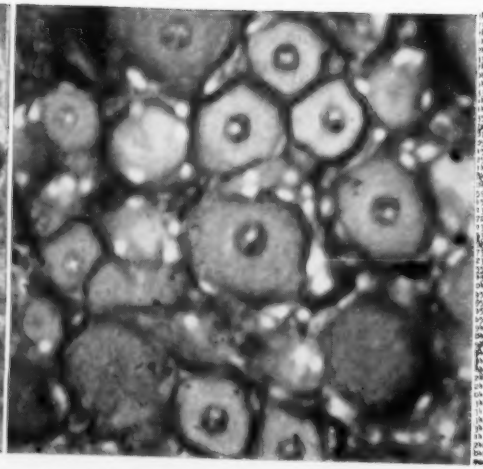


FIG. 5(b).—The same cells as in (a), exposed at 8–10 Å on Kodak spectrographic emulsion 649A.

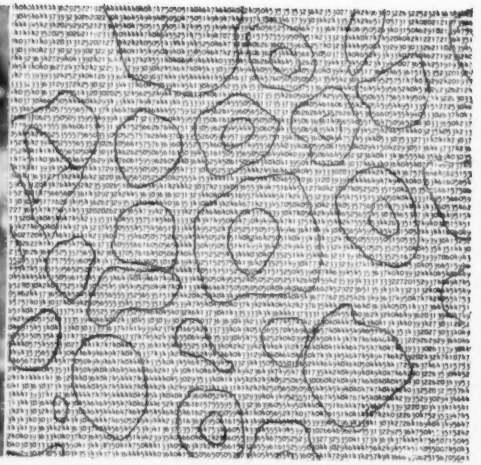


FIG. 5(c).—The picture in figures given by the apparatus.

facing p. 140

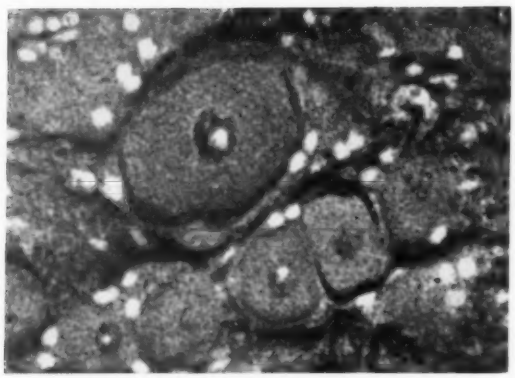


FIG. 6(a).—Carnoy fixed spinal ganglion cell, rabbit, exposed at 8–10 Å. Magnification 200×.

VOL
1
1956/

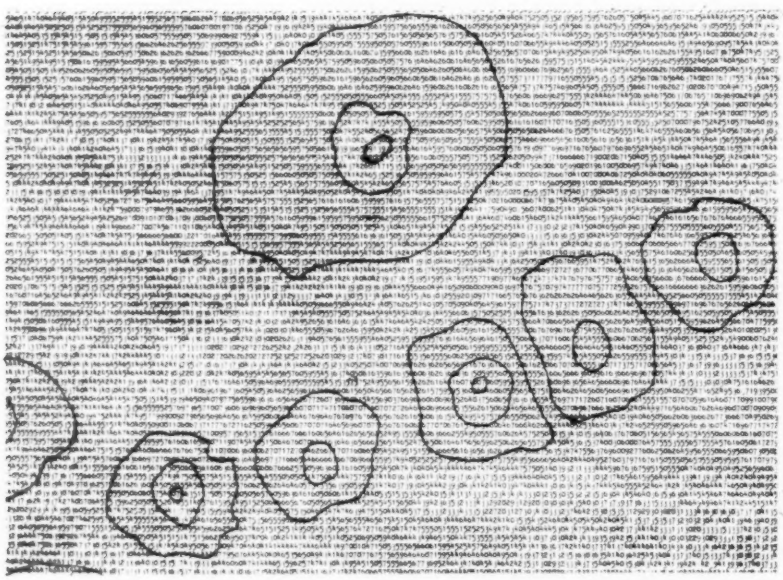


FIG. 6(b).—The picture in figures given by the apparatus of the same cells.

VOL.
1
56/57

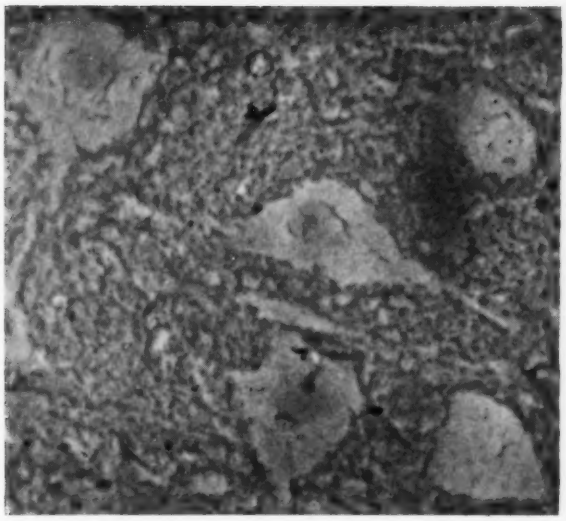


FIG. 7(a).—DEITER's cells, rabbit, exposed at 8–10 Å. Magnification 100×.

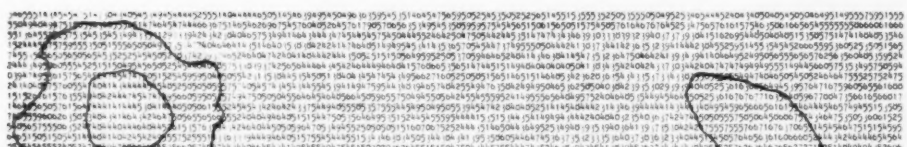


FIG. 7(b).—The picture in figures given by the apparatus of the same cells as in 7(a).

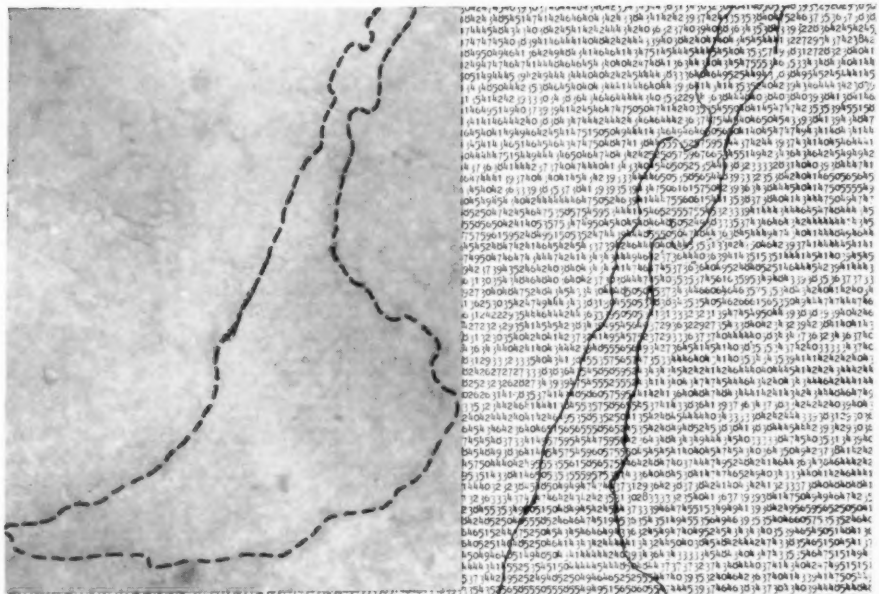


FIG. 8.—DEITER'S cell, rabbit, frozen, dried, and chloroform-extracted section together with the picture in figures. Magnification of the X-radiogram 200×.

VOL
1
1956/

above, reflect anything more than artefacts produced by the histological procedure. The gradients vary and are often directed from the cell periphery to the centre in those cells which are surrounded by a clear halo indicating a shrinkage effect. The fixation solutions dissolve part of the cell material and cause shrinkage, with the result that quantitative measurements of cell mass do not reflect conditions *in vivo*.

Detailed unicell analysis. This was performed on spinal ganglion cells at a magnification of 200× at the scanning plane (Fig. 6a, b). Optics: Zeiss apochromat 20×, n.a. 0·60, rabbit, Table 2.

TABLE 2

	Number of cells	Number of measurements	Mean of the mass in mg $10^{-9}/\mu^3$
Cytoplasm	5	1239	0·29
Nucleus	5	113	0·25
Nucleolus	2	14	0·39

Since a detailed analysis of this kind can be performed, it would appear to be of interest to get information also about the intracell variation. From Table 3 it is seen that the coefficient of variation on an average is less than 10%. On examining the distribution of the data no uniformity in the cytoplasm was observed. On the contrary as an expression of shrinkage the cell material was found to be more concentrated in certain parts of the cytoplasm, mostly at the periphery. This can no doubt be explained as an effect of the solutions used in the cytological procedure.

TABLE 3

	Cell No.	Number of measurements	Σ mass in mg $10^{-9}/\mu^3$	Mean of the mass in mg $10^{-9}/\mu^3$	σ	v%
Cytoplasm	1	683	169·258	0·25	0·030	12·2
	2	139	41·171	0·30	0·024	7·9
	3	155	50·994	0·33	0·027	8·2
	4	174	52·930	0·30	0·022	7·3
	5	88	25·616	0·29	0·027	9·35
Nucleus	1	44	9·168	0·21	0·032	15·3
	2	11	2·788	0·25	0·009	3·6
	3	15	4·198	0·28	0·014	5·0
	4	26	7·160	0·28	0·021	7·7
	5	17	4·250	0·25	0·013	5·2
Nucleolus	1	8	3·145	0·39	0·035	8·8
	4	6	2·282	0·38	0·029	7·7

FRESH, FROZEN NERVE CELL MATERIAL

DEITER's cells from rabbit were used, and Fig. 7(a) and (b) show as an example an X-radiogram exposed at 8–10 Å, and the picture in figures of the frozen, dried, and chloroform-extracted section. Magnification at the scanning plane 100×. Optics: Zeiss apochromat 10×, n.a. 0·35. The sections were exposed before and after the treatment with chloroform in order to get the lipid fraction extracted by that solvent from the various cell structures. In these measurements particular attention was paid also to obtaining numerical information on the dendrites.

Multicell analysis—Even if the number of cells analysed is small the figure for the coefficient of variation is high enough to motivate a closer look on the distribution.

TABLE 4

	Number of cells	Number of measurements	Mass expressed in mg $10^{-9}/\mu^3$						Lipid fraction in % of dry tissue
			before $CHCl_3$			after $CHCl_3$			
			mean	σ	v%	mean	σ	v%	
Cytoplasm	16	5248	0·25	0·034	13·5	0·20	0·05	25·0	19·3

It was found that the mass values were grouped around two means, 0·23 and 0·29 mg . $10^{-9}/\mu^3$ respectively. A t analysis showed the difference to be significant ($P < 0{\cdot}001$). This may give a clue for further investigations. The morphology and staining properties of the cells belonging to the Deiter's nucleus do not permit a classing of cells in subgroups. The finding reported here, however, does not exclude a subdivision in at least two groups of cells different in chemical and physical respects.

DETAIL UNICELL ANALYSIS

Fig. 8 shows as an example one of the Deiter's cells exposed at 8–10 Å, the section of which includes part of a dendrite. Magnification 200× at the scanning plane. Optics: Zeiss apochromat 20, n.a. 0·60. Note the uniformity of the X-ray absorption throughout the cell. The mass of the nucleus is approximately that of the cytoplasm. Hence, the nucleus does not show up in the radiogram as an area of less absorption as in a stained section.

TABLE 5

	Number of cells	Number of measurements	Mean of the mass in mg $10^{-9}/\mu^3$		Lipid fraction in % of dry tissue
			before $CHCl_3$	after $CHCl_3$	
Cytoplasm	4	2849	0·27	0·22	17
Dendrite	2	424	0·27	0·20	26
Nucleus	4	197	0·20	0·17	20

It is of considerable interest to examine the result of the detail analysis of this fresh and frozen material which is as close to the native state from a cytochemical viewpoint as it at present is possible to obtain Particularly the intracellular distribution of the cell material, including the variation of the mass, the presence of any gradients, and the mass concentration of the dendrites, are of biological interest.

TABLE 6

	Cell number	Number of measurements	Σ mass in mg $10^{-9}/\mu^3$	Mean of the mass in mg $10^{-9}/\mu^3$	σ	$v\%$
Cytoplasm	1	1223	369·2	0·30	0·040	13·1
	2	854	230·9	0·27	0·028	10·4
	3	69	21·2	0·31	0·036	11·6
	4	57	17·4	0·31	0·021	6·8
Dendrite	1	235	66·6	0·28	0·031	10·9
	2	132	36·9	0·28	0·034	12·2
Nucleus	2	24	7·0	0·29	0·018	6·2

As is seen from Tables 5 and 6 the mass value for the nucleus is approximately the same as that of the cytoplasm. With respect to the intracellular mass distribution, it is of interest to ascertain whether gradients exist, and if so, their relative magnitude. Judged from about 3000 values taken of the large Deiter's cells there exists a gradient in the cell material extending from the nucleus to the cell periphery and amounting to around 25% expressed in per cent of the mass near the nucleus. It can be noted that this gradient is less prominent in the fresh, frozen, and dried sections before the chloroform extraction.

The amount of dry substance in the dendrites is nearly as high as that for the cytoplasm, i.e. 0·28 mg $\times$ $10^{-9}/\mu^3$. That the dendrite has so high a concentration of cell material may be linked up with some observations on the importance of the dendrites Recent investigations have indicated that a considerable part of activity of the nerve cells is localized to the dendrites (LOWRY, 1955) which can also be characterized functionally.

As is seen from Table 5, the lipid fraction of the dendrites seems to be high. This calls for a comment The volume of the dendrites does not occupy the whole volume of the part of the section studied. Thus above and below the dendritic part lipid-rich material adds its mass value per volume unit to that of the dendritic structure. Therefore it can be supposed that the lipid fraction of the dendrites constitutes less than the 26% obtained. How significant the values of the dendrite are depends on to what extent the section studied consists solely of dendritic tissue.

SUMMARY

A scanning and computing cell analyser for quantitative purposes is described, including some new modifications constructed after the original description.

The apparatus performs a calculation of 12,000 transmission or density values in a microscopic field. The values are stored with maintained spatial localization.

The result can be obtained as (1) a single value representing the integral of the individual values recorded within the area scanned, or (2) as a picture in density values. Thus the apparatus allows the recording of an average density value per cell section, or it can be used to obtain many density values on a number of cells in a short time. The calculation of the 12,000 values takes four minutes.

The resolving power depends on the optical system used. An area of 0·2 μ^2 for an individual measurement can be calculated if a light microscope is used The sources of error of the apparatus have been studied and the total error of the performance amounts to 2·5%.

The apparatus has been applied to the evaluation of X-radiograms of nerve cell sections taken at 8–10 Å to compute the amount of dry substance and the amount of chloroform-soluble lipids. In all 25,000 measurements were performed, and the amount and distribution of cell mass was computed. In fresh, frozen, and dried cell sections a concentration gradient was found directed from the nuclear membrane towards the cell periphery. The findings on fixed nerve cells showed that it is inadvisable to use such material for gradient determinations or for distribution studies. High values of both cell mass and also lipids were found in the dendrites. This is noteworthy in view of the functional activity ascribed to these parts of the neuron.

REFERENCES

BOURGHARDT S., HYDÉN H., and NYQUIST B. (1955) A computing microphotometer for cell analyses. *J. of Sci. Instr.* **32,** 186.

BRATTGÅRD S.-O. and HYDÉN H. (1954) The composition of the nerve cell studied with new methods. *Int. Rev. of Cytol.* **3,** 455.

DEELEY E. M. (1955) An integrating microdensitometer for biological cells. *J. of Sci. Instr.* **32,** 263.

EDSTRÖM J.-E. (1953) Quantitative determination of ribonucleic acid from individual nerve cells. A new method for quantitative determinations. *Biochim. et Biophys. Acta* **12,** 361.

EDSTRÖM J.-E. (1953) Nucleotide analysis on the cyto-scale. *Nature* **172,** 809.

EDSTRÖM J.-E. and HYDÉN H. (1954) Ribonucleic analysis of individual nerve cells. *Nature* **174,** 128.

GODLOVE T. F. and WADEY W. G. (1954) Photocathode uniformity and resolution of scintillation spectrometers. *The Rev. of Sci. Instruments* **25,** 1.

HALLÉN O. (1956) On the cutting and thickness determination of microtome sections. A study in quantitative cytology. *Acta Anatomica, suppl.* **26,** 25.

HYDÉN H. (1955) The chemistry of single neurons: a study with new methods, in *Biochemistry of the Developing Nervous System.* Edited by H. WAELSCH, Academic Press, New York, p. 357.

LOMAKKA G. (1955) A recording function transformer and integrator for microspectrographic use. *Exp. Cell. Res.* **9,** 434.

LOWRY O. (1955) in *Biochemistry of the Developing Nervous System.* Edited by H. WAELSCH, Academic Press, New York, p. 350.

THORNBURG W. (1956) Pulse counting photometer for microspectroscopy. *Rev. of Sci. Instr.* **27,** No. 2, 99.

TOLLES W. E. (1955) The cytoanalyzer—an example of physics in medical research. *Transactions of the New York Acad. of Sci. Ser. II* **17,** 250.

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 145–149, Pergamon Press Ltd., London.

THE ACTION OF CHLORPROMAZINE AND RESERPINE ON BRAIN ENZYME SYSTEMS*

JOSEPH BERNSOHN, IZOLINA NAMAJUSKA, and BENJAMIN BOSHES
Neuropsychiatric Research Laboratory, V. A. Hospital, Hines, Illinois,
and Northwestern University Medical School, Chicago, Illinois

(*Received* 7 *May* 1956)

STUDIES on the mechanism of action of chlorpromazine have indicated that the drug can act as an uncoupling agent of oxidative phosphorylation and also as an inhibitor of ATP-ase and cytochrome oxidase (ABOOD, 1955; BERNSOHN, NAMAJUSKA, and COCHRANE, 1956a). The relationships that may exist between these mechanisms are imperfectly understood. The large variety of compounds which can depress the P : O ratio suggests that there is no specific correlation between this inhibitory action and physiological function. Also, there is no apparent explanation of how the inhibition of cytochrome oxidase and/or of ATP-ase can provide a basis for the action of chlorpromazine, since other cytochrome oxidase inhibitors, viz. cyanide, azide, and other ATP-ase inhibitors viz, fluoride, do not possess pharmacological properties akin to chlorpromazine. However, the kinetics of the inhibition, as well as the binding sites of the inhibitor to the enzyme and the accessibility of the target enzyme to the organic inhibitor, may vary sufficiently to impart properties to the drug which are different from those of other inhibitors of these enzymes.

Since both reserpine and chlorpromazine can produce ataraxia, hypotension, and hypothermia, it is of interest to determine whether the two drugs have any similar mode of action. CENTURY and HORWITT (1956) have shown that a synergistic effect of reserpine on chlorpromazine occurs in inhibiting oxidative phosphorylation, though the molar concentrations of the drugs are in excess of that expected *in vivo* on a uniform distribution basis. On the other hand, BRODY, SHORE, and PLETSCHER (1956) have found that chlorpromazine does not act by releasing serotonin from brain tissue, whereas this has been postulated as a basis for the clinical effects of reserpine. While a common mechanism may be looked for, dissimilarity in behaviour is also of interest, since each drug has properties which are confined to only one of the compounds. For example, reserpine does not possess the barbiturate-potentiating property of chlorpromazine (STEPHAN, BOURGEOIS-GAVARDIN, and MARTIN, 1955). In this work, further studies have been made on the effect of these drugs on the cytochrome oxidase and ATP-ase systems.

METHODS

The methods for the preparation of mitochondria and the conditions employed for the assay systems were the same as previously reported (BERNSOHN, NAMAJUSKA, and COCHRANE, 1956b). Purified cytochrome oxidase was prepared by the procedure of WAINIO, COOPERSTEIN, KOLLEN, and EICHEL (1948) from pig heart, since attempts to obtain a purified cytochrome-oxidase preparation from brain by this method proved unsuccessful, probably owing to the high lipid content of brain

* This work was supported in part by a grant from the Mental Health Fund of the Illinois Department of Welfare.

tissue. In one experiment where the effects of chlorpromazine and reserpine in combination were studied, the drugs were added to the main compartment of the vessel; in another series of experiments one of the drugs was placed in the main compartment and the other compound tipped in from the side-arm after respiration had proceeded for 15 minutes. Conditions for each assay system are noted in the tables.

Chlorpromazine was donated by Smith, Kline and French Laboratories and reserpine by the Research Department of Ciba Pharmaceutical Company. The reserpine supplied was in a lyophilized form and soluble in the buffer employed, thus obviating any difficulties arising from insolubility. The preparation used in this work was made by the addition of phosphoric acid to reserpine prior to lyophilization. A molecular weight of 608 was assigned to reserpine and the assumption was made that it was a pure product and in the free form. Thus the calculated molarities were maximal values.

RESULTS

A comparison of the inhibitory properties of reserpine and chlorpromazine toward the ATP-ase and cytochrome oxidase systems is presented in Table 1. It is apparent

TABLE 1.—INHIBITION OF CYTOCHROME OXIDASE AND ATP-ASE IN RAT BRAIN MITOCHONDRIA BY CHLORPROMAZINE AND RESERPINE

A. Cytochrome oxidase			B. ATP-ase		
Drug M	O_2* μmoles	Inhibition %	Drug M	P* μmoles	Inhibition %
		Chlorpromazine			
0	48	—	0	37	—
5×10^{-4}	5	89	5×10^{-4}	16	59
1×10^{-4}	36	26	—	—	—
5×10^{-5}	43	11	—	—	—
		Reserpine			
0	47	—	0	37	—
1×10^{-3}	16	66	8×10^{-4}	16	56
5×10^{-4}	36	23	5×10^{-4}	30	19

* Per 100 mg fresh tissue per hour.

A. Reaction Mixture (cytochrome oxidase): Cytochrome c, 8×10^{-5} M; Na ascorbate, $1{\cdot}1 \times 10^{-2}$ M; $AlCl_3$, 4×10^{-4} M; phosphate buffer, 0·04 M, *p*H 7·4; incubation time—30 min; final volume—3·0 ml; mitochondria equivalent to 20 mg fresh tissue.

B. Reaction Mixture (ATP-ase): K_2ATP, 3×10^{-3} M; $MgSO_4$, 3×10^{-3} M; veronal buffer, 0·023 M, *p*H 7·4; incubation time—15 min; final volume—2·6 ml; mitochondria equivalent to 40 mg fresh tissue.

that both drugs have an inhibitory action on these enzyme systems. However, at equivalent molar concentrations chlorpromazine is a more potent inhibitor of both systems than is reserpine. In addition, under these experimental conditions, chlorpromazine is more inhibitory toward the cytochrome-oxidase system than toward ATP-ase, since at a concentration of 5×10^{-4} M the inhibition is 89% and 23% respectively, while reserpine inhibits both systems equally. The inhibition at a concentration of 1×10^{-3} M is 66% for cytochrome oxidase and 56% for ATP-ase. At 5×10^{-4} M, the values are more nearly equal.

To define the mechanism of inhibition more adequately, some experiments were performed in which the effect of the drugs could be determined when both were

present in the same reaction mixture. The results obtained with cytochrome oxidase are shown in Fig. 1a and 1b. In Fig. 1a the inhibition of cytochrome oxidase by reserpine and chlorpromazine alone and in combination is shown. It is evident that the drugs when combined have no additive effect, but inhibit cytochrome-oxidase activity to a value which is intermediate between the values obtained when the drugs are used alone, at the concentrations studied. This effect is also obtained when the reaction in the presence of one inhibitory drug is allowed to proceed and the second inhibitory drug is tipped in from the side-arm after a suitable interval (Fig. 1b). At the 15-minute interval (marked by arrows), the second drug was tipped into the main compartment, where the first drug had already established the inhibitory slope. On addition of the second drug, the velocity of the reaction was altered. The dotted lines

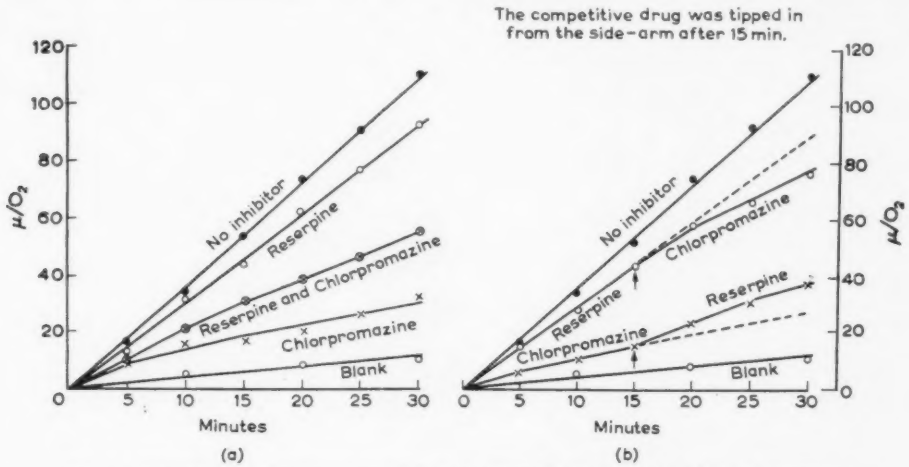


FIG. 1.—Inhibition of cytochrome oxidase by chlorpromazine ($2{\cdot}5 \times 10^{-4}$ M) and reserpine (5×10^{-4} M).

indicate the expected velocity if the original reaction had been allowed to proceed without any modification. It can be seen that in both cases the addition of the second drug modified the velocity of the reaction to a value intermediate between the two original velocities. The action of the drug seems to be immediate, within the time

TABLE 2.—EFFECT OF CHLORPROMAZINE AND RESERPINE ON ATP-ASE IN RAT BRAIN MITOCHONDRIA

Drug M	P μmoles/100 mg/15 min	Inhibition %
——	9·3	—
Chlorpromazine, 5×10^{-4}	3·9	59
Reserpine, 5×10^{-4}	7·5	19
Reserpine, 8×10^{-4}	4·1	56
Chlorpromazine, 5×10^{-4} Reserpine, 5×10^{-4}	3·6	62
Chlorpromazine, 5×10^{-4} Reserpine, 8×10^{-4}	2·9	69

Reaction mixture: Same as Table 1.

interval of the measurements, and would confirm previous observations that prior incubation of enzyme and inhibitor did not increase the inhibitory effect.

The effect of chlorpromazine and reserpine in combination on ATP-ase activity was also determined. The results are shown in Table 2. In contrast to their effect on cytochrome oxidase, a synergistic effect was observed here, since the addition of reserpine (8×10^{-4} M) to chlorpromazine increased the inhibition found with the latter drug alone from 59% to 69%.

While the inhibitory effects found in these studies are apparent, the molar concentrations of the drugs required to produce them are in excess of the pharmacological dose. The action of the drugs on a purified cytochrome-oxidase preparation was therefore studied to ascertain whether removal of inert proteins would have any effect on the degree of inhibition. Using the procedure of WAINIO *et al.* (1948) a partially purified preparation was obtained which gave a Q_{O_2} value of 722 μmoles/mg nitrogen/hr. Since the original pig heart homogenate had a Q_{O_2} value of 62·2 μmoles/mg nitrogen/hr, a purification of 11·6 times was achieved. The inhibitory effect of chlorpromazine on this product was about fivefold greater, since the pI_{50}* for the homogenate was found to be 3·7 and for the preparation pI_{50} was equal to 4·4 (Table 3). In contrast to chlorpromazine, no augmentation of the inhibitory effect was observed on this preparation with reserpine. The inhibition of the pig heart

TABLE 3.—CYTOCHROME OXIDASE INHIBITION BY CHLORPROMAZINE AND RESERPINE IN PIG HEART

Concentration M	Homogenate		Purified preparation	
	Oxygen μmoles/100 mg/hr	Inhibition %	Oxygen μmoles/hr	Inhibition %
Chlorpromazine				
0	354	0	19	—
5×10^{-4}	39	89	1	93
$2{\cdot}5 \times 10^{-4}$	161	55	2	90
1×10^{-4}	225	36	5	73
5×10^{-5}	300	15	8	55
$2{\cdot}5 \times 10^{-5}$	353	0	13	30
1×10^{-5}	354	0	16	12
5×10^{-6}	—	—	17	8
Reserpine				
0	348	—	18	—
5×10^{-4}	229	34	11	36
1×10^{-4}	338	3	15	16

Reaction mixture: Same as Table 1, except 0·2 ml homogenate (1 : 40) or 0·2 ml of purified preparation (1 : 25) were used instead of mitochondria.

* pI_{50} = logarithm of the reciprocal of the inhibitor concentration required to produce 50% inhibition of the enzyme.

homogenate was 34% compared with 36% for the partially purified cytochrome oxidase.

DISCUSSION

While the desirability of finding an explanation for the action of ataractic drugs is obvious, the difficulty of establishing a mechanism of action for reserpine and chlorpromazine is evident in view of the multiple biological properties of the two drugs. In this study on the effect of these two compounds on cytochrome oxidase and ATP-ase, some common properties were observed. Both drugs inhibit the two enzyme systems, but the concentrations required are far in excess of the pharmacological level. Without exploring the complex situation involved in relating the concentration *in vitro* to the *in vivo* situation, it was felt that the role of the binding of the drug by cell components not involved in the system under study should be evaluated. The partially purified cytochrome-oxidase preparation gave data which indicated that the binding effect may be considerable for chlorpromazine, but not for reserpine. Since the usual clinical dosage of reserpine is smaller than that employed with chlorpromazine, it would seem that, if the pharmacological effect of reserpine is mediated through the cytochrome-oxidase and ATP-ase systems, it must have an action confined to a localized area in the brain.

It seems that both reserpine and chlorpromazine inhibit cytochrome oxidase by attacking a similar site in the system, whereas the inhibition of ATP-ase by the two drugs occurs by two different mechanisms. It would seem that the action of chlorpromazine in inhibiting the two enzyme systems could afford a mechanism of action of this drug, whereas this is not likely for reserpine.

SUMMARY

The inhibitory properties of chlorpromazine and reserpine toward cytochrome oxidase and ATP-ase have been further investigated. The inhibition of cytochrome oxidase by the two drugs is different from the inhibition of ATP-ase, which is synergistic. Studies on a partially purified cytochrome-oxidase preparation indicate that the binding of chlorpromazine by noncytochrome oxidase components is an important factor in relating inhibitory effect to drug concentration, whereas this is not true for reserpine.

REFERENCES

ABOOD L. G. (1955) *Proc. Soc. Exp. Biol., N.Y.* **88,** 688
BERNSOHN J., NAMAJUSKA I., and COCHRANE L. S. G. (1956a) *Arch. Biochem. Biophys.* **62,** 15.
BERNSOHN J., NAMAJUSKA I., and COCHRANE L. S. G. (1956b) *Proc. Soc. Exp. Biol., N.Y.* **92,** 201.
BRODY B. A., SHORE P. A., and PLETSCHER A. (1956) *Science* **123,** 992.
CENTURY B., and HORWITT M. K. (1956) *Proc. Soc. Exp. Biol. N.Y.*, **91,** 493.
STEPHAN C. R., BOURGEOIS-GAVARDIN M., and MARTIN R. C. (1955) *Ann. N.Y. Acad. Sci.* **61,** 236.
WAINIO W. W., COOPERSTEIN S. J., KOLLEN S., and EICHEL B. (1948) *J. Biol. Chem.* **173,** 145.

Journal of Neurochemistry, 1956, Vol. 1, pp. 150–152, Pergamon Press Ltd., London.

THE DEPRESSION OF PHOSPHOLIPID TURNOVER IN BRAIN TISSUE BY CHLORPROMAZINE

G. B. ANSELL and H. DOHMEN*
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(*Received* 9 *September* 1956)

VOL
1
1956/

IT has been previously reported that the turnover of the phospholipid phosphorus of the brain is affected by changes in the physiological state (DAWSON and RICHTER, 1950). However, little information is available about changes in the different phospholipid fractions. In the course of studies on the effect of various experimental conditions on the metabolism of phospholipids *in vivo*, the effect of the tranquillizing drug, chlorpromazine (Largactil), was tested. The present paper reports the effect of this drug on the turnover of total rat brain phospholipid and of the choline- and ethanolamine-containing phospholipids.

METHODS

Young rats (*ca.* 50 g body weight) were injected intramuscularly with chlorpromazine (20 mg/kg body weight) and after 30 min were given 3 mc ^{32}P/kg body weight in 11·6 mg NaH_2PO_4/kg body weight in sterile, isotonic solution by intraperitoneal injection. The chlorpromazine-treated rats showed a marked diminution in activity throughout the isotopic-exchange period in comparison with littermate control animals which received only ^{32}P orthophosphate. At 3 hours after the injection of ^{32}P, the animals were decapitated, the brains were homogenized with 10% (w/v) ice-cold trichloroacetic acid solution, and a clear tissue extract was obtained by centrifuging at 0° at 25,000 × g. The tissue was washed twice with 5% (w/v) trichloroacetic acid solution at 0° and the combined extracts plus washings were taken as the acid-soluble phosphorus fraction. After washing the precipitated tissue twice with water, it was dried with acetone; the acetone washings were combined with the solution of total brain lipid obtained by extracting the dried tissue with chloroform/methanol (1 : 1 v/v) in a Soxhlet apparatus for 4 hours. The specific radioactivity of the phospholipid P and acid-soluble P was measured after oxidation with perchloric acid to inorganic orthophosphate. The specific radioactivities of individual phospholipids were measured by the method of DAWSON (1954), except that the lipid solution was washed twice with 2 volumes of 0·25 M magnesium chloride solution before saponification.

RESULTS

The effect of chlorpromazine on the uptake of radioactive phosphorus into the phospholipids of the brain is shown in Table 1, in which the ratio of the specific radioactivity of the phospholipid phosphorus to that of the acid-soluble phosphorus is used as a measure of the rate of phospholipid synthesis (cf. DAWSON and RICHTER, 1950). The specific radioactivity of the acid-soluble phosphorus showed no consistent change as a result of chlorpromazine administration. It can be seen however, that after a 3-hour exchange period, the average rate of phospholipid synthesis in the brain of the chlorpromazine-treated animals was only 44% of that of the controls. Phosphatidylcholine synthesis was reduced to 37% and phosphatidylethanolamine

* Present address: Physiologisch-Chemisches Institut der Universität, Köln, Germany.

synthesis to 50% of the normal. A decrease of about 40% was noted in the turnover of phosphatidylserine, into which the uptake of labelled phosphorus was very small in the normal animals. These effects of chlorpromazine are comparable in size with the depression of phospholipid turnover obtained in animals during deep pentothal anaesthesia (DAWSON and RICHTER, 1950; ANSELL and DOHMEN, 1957).

TABLE 1.—EFFECT OF CHLORPROMAZINE ON PHOSPHOLIPID TURNOVER IN WHOLE RAT BRAIN

	Specific radioactivity ratios × 1000		
	Total phospholipid P / Acid-soluble P	Phosphatidylcholine / Acid-soluble P	Phosphatidylethanolamine P / Acid-soluble P
Normal	39·4 ± 1·7 (6)	49·8 ± 2·8 (6)	26·0 ± 2·2 (6)
Chlorpromazine	17·4 ± 3·1 (6)	18·3 ± 5·1 (6)	12·9 ± 2·4 (6)

These results were obtained on two separate litters of six animals, in each of which three were normals and three were treated with chlorpromazine. Means are given ± standard deviations

It should be noted that in the method of DAWSON (1954) the glycerylphosphorylethanolamine separated for the measurement of the specific radioactivity of the phosphatidylethanolamine may be partly derived from the ethanolamine plasmalogen, since this could yield a saponifiable lysokephalin when the tissue is homogenized with dilute acid (the choline-containing plasmalogen of heart muscle is known to yield a lysolecithin in the presence of dilute acetic acid) (KLENK and DEBUCH, 1955). This dual source of glycerylphosphorylethanolamine might well be significant in brain tissue where the ethanolamine plasmalogen occurs in greater concentration than the corresponding ester phospholipid (KLENK and BÖHM, 1951); but it would be insignificant in liver tissue, which contains a negligible amount of the former (KLENK and DOHMEN, 1955).

From experiments *in vitro* it is now well established that phospholipid synthesis in brain tissue requires conditions in which the rate of oxidative phosphorylation is high (DAWSON, 1953; STRICKLAND, 1954). Since chlorpromazine is believed to uncouple oxidative phosphorylation in isolated brain systems (ABOOD, 1955; CENTURY and HORWITT, 1956), it is possible that this may account for the effect observed *in vivo*.

While this work was in progress, WASE, CHRISTENSEN, and POLLEY (1956) reported that chlorpromazine depresses the total phospholipid synthesis in the cerebral cortex, but stimulates, or has no effect on, synthesis in other parts of the brain. It is difficult to compare their results with those in Table 1, because WASE *et al.* determined the specific radioactivity of the lipids after exchange periods of 24–48 hours and did not relate it to that of the acid-soluble fraction. The experiments carried out here indicate that the ratio $\frac{\text{specific radioactivity of phospholipid P}}{\text{specific radioactivity of acid-soluble P}}$ after a 3-hour exchange period is reduced by chlorpromazine in the cerebral cortex-medulla, pons, white matter, and cerebellum (Table 2). These experiments give little evidence of any significant differences in the different parts of the brain.

SUMMARY

1. Chlorpromazine depressed the turnover of phospholipids in brain tissue *in vivo*. The effect on turnover is more marked for phosphatidylcholine than for phosphatidylethanolamine.

TABLE 2.—THE EFFECT OF CHLORPROMAZINE ON PHOSPHOLIPID TURNOVER IN DIFFERENT PARTS OF THE RAT BRAIN

	Ratio $\frac{\text{specific radioactivity phospholipid P}}{\text{specific radioactivity acid-soluble P}} \times 1000$	
	Normal	Chlorpromazine-treated
Cortex	34·1	15·7
Cerebellum	31·6	20·3
White matter	33·5	20·8
Pons and medulla	33·2	19·7

Parts from three experimental and three control animals were pooled. The six rats were littermates.

2. A depression in turnover was observed uniformly in cerebal cortex, pons, medulla, white matter, and cerebellum.

Acknowledgement—The authors are grateful to Dr. D. RICHTER for his advice and encouragement. One of us (H. D.) is indebted to the Rockefeller Foundation for a personal grant, and we thank the Medical Research Council for a grant in aid of expenses. We thank Miss ANNE PHILLIPS for skilled assistance.

REFERENCES

ABOOD L. G. (1955) *Proc. Soc. Exp. Biol., N.Y.* **88,** 688.
ANSELL G. B. and DOHMEN H. (1957) (in preparation).
CENTURY B. and HORWITT M. K. (1956) *Proc. Soc. Exp. Biol., N.Y.* **91,** 493.
DAWSON R. M. C. (1953) *Biochem. J.* **55,** 507.
DAWSON R. M. C. (1954) *Biochim. Biophys. Acta.* **14,** 374.
DAWSON R. M. C. and RICHTER D. (1950) *Proc. Roy. Soc. B.* **137,** 252.
KLENK E. and BÖHM P. (1951) *Hoppe-Seyl. Z.* **288,** 98.
KLENK E. and DEBUCH H. (1955) *Hoppe-Seyl. Z.* **299,** 66.
KLENK E. and DOHMEN H. (1955) *Hoppe-Seyl. Z.* **299,** 248.
STRICKLAND K. P. (1954) *Canad. J. Biochem. Physiol.* **32,** 50.
WASE A. W., CHRISTENSEN J., and POLLEY E. (1956) *Arch. Neurol. Psychiat. Chicago.* **75,** 54.

VOL 1 1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 153–158. Pergamon Press Ltd., London.

SOME METABOLIC ASPECTS OF LEÃO'S SPREADING DEPRESSION

J. BUREŠ
Institute of Physiology, Czechoslovak Academy of Sciences, Prague

(*Received* 22 *August* 1956)

VOL. 1 56/57

THE metabolism of brain slices under the influence of increased potassium chloride concentration and electrical stimulation has been thoroughly studied in recent years (DIXON, 1949; MCILWAIN, 1954; ELLIOTT, 1955). *In vitro*, 0·1 M potassium chloride increases the Q_{O_2} and aerobic glycolysis and decreases "high-energy" phosphorus (~P) content of brain slices when glucose, fructose, pyruvate, or lactate are added to the medium as substrate. The question then arises as to whether there is a physiological correlate of these metabolic reactions. *In vivo* 0·134 M potassium chloride applied to the cortical surface of the rabbit brain elicits spreading depression, as was shown for the first time by LEÃO and MORISON (1945). This reaction consists of a slowly expanding wave of depression of electrical activity moving through the cortex at a rate of 3–6 mm/min. The ECoG depression is accompanied by a striking variation of the cortical steady potential, an initial surface negativity (5–10 mV) followed by a more prolonged positivity. The gross character of the changes accompanying spreading depression, the low rate of propagation, the resistance to general anaesthesia (VAN HARREVELD and STAMM, 1953), the sensitivity to asphyxia (LEÃO, 1947) and other characteristic features, suggest that spreading depression is a neurochemical reaction.

Thus the possibility must be considered, that the metabolic effects of potassium ions observed in brain slices participate in spreading depression. The purpose of this paper is to test the hypothetical analogy between these *in vivo* and *in vitro* reactions of cortical tissue.

METHODS

The experimental procedure used has been described elsewhere (BUREŠ and KOŠTOJANC, 1955; BUREŠ and BUREŠOVÁ, 1956). All experiments were carried out on rats anaesthetized with allobarbitone (Dial) (40 mg/kg i.p.). Bipolar ECoG was picked up by steel needle electrodes fixed in skull bones and registered by an ink-writing six-channel EEG apparatus (Ediswan). Steady potential changes were recorded by calomel cell electrodes applied directly to the cerebral cortex through small trephine holes (ϕ 2·5 mm) over frontal areas of each hemisphere. The chopper technique (GOLDRING and O'LEARY, 1951) was used for recording the slow voltage variations directly in one of the EEG channels. Larger trephine holes (ϕ 5 mm) over occipital parts of the hemispheres were used for chemical stimulation of the cortex by means of small (2 × 2 mm) pieces of filter paper soaked with the solutions studied. The method of threshold determination is illustrated in Table 1, which gives complete data for 2,4-DNP. The ED 50 was computed by probit analysis (FINNEY, 1950) or by the moving averages method (WEIL, 1952).

TABLE 1.—DETERMINATION OF THE THRESHOLD FOR 2,4-DINITROPHENOL

2,4-DNP M	$2{\cdot}75 \times 10^{-5}$	$5{\cdot}41 \times 10^{-5}$	$2{\cdot}72 \times 10^{-4}$	$5{\cdot}41 \times 10^{-4}$	$5{\cdot}41 \times 10^{-3}$
n	8	14	9	9	4
Spreading depression	0	5	3	6	4
No spreading depression	8	9	6	3	0
Percentage of s.d.	0	35·7	33·4	66·7	100

RESULTS

There are a number of other substances besides potassium ions that elicit similar metabolic reactions *in vitro*—ammonium chloride, glutamine, 2,4-dinitrophenol, veratrine (WEIL-MALHERBE, 1938; GHOSH and QUASTEL, 1954; WOLLENBERGER, 1955). If the assumption is correct that the metabolic reactions described above form the chemical basis of spreading depression, these substances should evoke spreading depression when applied locally to the cortex. We have, therefore, attempted to determine the threshold concentration for each of these drugs. Several (3 to 5) different concentrations, from subminimal to supramaximal were used for each substance.

Table 2 gives the computed threshold concentrations (ED 50). In good agreement with the postulated analogy between *in vitro* and *in vivo* experiments all the drugs tested caused spreading depression in relatively low concentrations. On the other

TABLE 2.—COMPUTED THRESHOLD CONCENTRATION (ED 50) FOR DIFFERENT COMPOUNDS

	ED 50	log ED 50 ± SD
KCl	0·080 M	$-1{\cdot}097 \pm 0{\cdot}024$
NH_4Cl	0·125 M	$-0{\cdot}903 \pm 0{\cdot}023$
L-glutamine	0·077 M	$-1{\cdot}117 \pm 0{\cdot}047$
2,4-DNP	0·0002 M	$-3{\cdot}682 \pm 0{\cdot}263$
Veratrine	approx. 0·01–0·1%	

hand, spreading depression could not be elicited by a great number of other substances, e.g. by NaCl, $NaHCO_3$, Na_2HPO_4, $MgCl_2$, $CaCl_2$, acetylcholine, methionine, glutathione, urea, glucose, sucrose, etc.

It was shown by various authors that citrate, α-ketoglutarate, glutamate, and succinate, if added to the suspension medium, prevent the increase of Q_{O_2} in brain slices caused by KCl or electrical stimulation (LIPSETT and CRESCITELLI, 1950; MCILWAIN, 1954). Similar results have been obtained more recently with malonate (KIMURA and NIWA, 1953). By the technique already described we attempted to reproduce these results *in vivo*. Spreading depression was evoked by 1% KCl solutions which at the same time contained variable amounts of the sodium salts (pH 7) of the organic acids studied. Fig. 1 gives the results of such an experiment with α-ketoglutarate and Table 3 the mean values of ED 50 for the concentration ratios

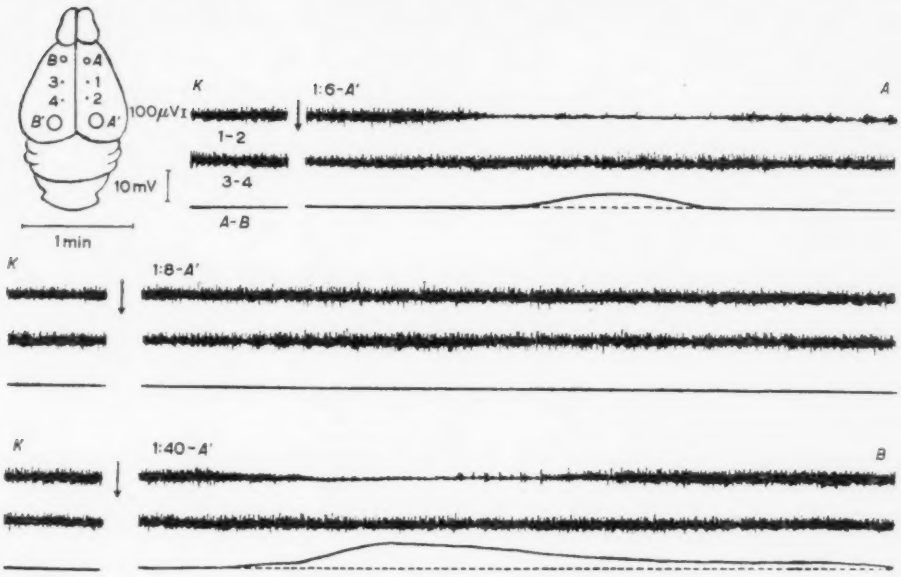


FIG. 1.—Antagonism between potassium and α-ketoglutarate in the initiation of spreading depression.
1–2, 3–4—ECoG recorded with steel-needle electrodes.
A–B—slow potential change recorded with calomel-cell electrodes between *A* and *B*. An upward deflection indicates increased negativity of *A*.
The arrow indicates the application of 1% KCl solution which contains also 6% (first record) or 8% (second record) of α-ketoglutaric acid, neutralized by NaOH. In the last record 1% KCl in 40% glucose was applied. Calibration: 100 μV for ECoG, 10 mV for steady potential.

TABLE 3.—THE PREVENTION OF SPREADING DEPRESSION BY OXIDIZABLE SUBSTANCES

	ED 50 $\frac{[acid]}{[K^+]}$	log ED 50 ± SD
1% KCl + citrate	2·57	0·710 ± 0·021
1% KCl + α-ketoglutarate	2·97	0·473 ± 0·038
1% KCl + succinate	9·97	0·999 ± 0·018
1% KCl + malonate	0·940	−0·027 ± 0·079

[acid]/[K^+] preventing the development of spreading depression. On the other hand glucose, even in 40% solution and saturated NaCl, does not modify the effect of 1% KCl. The hypothetical analogy between spreading depression and the potassium effect *in vitro* was considerably extended by these experiments.

A further experimental approach to the problem was the application of metabolic inhibitors. It has been demonstrated that besides 2,4-DNP, the inhibitors NaN_3, NaCN, and NaF also elicit a typical spreading depression when applied locally to the cerebral cortex (Fig. 2). Table 4 gives the mean threshold concentrations for

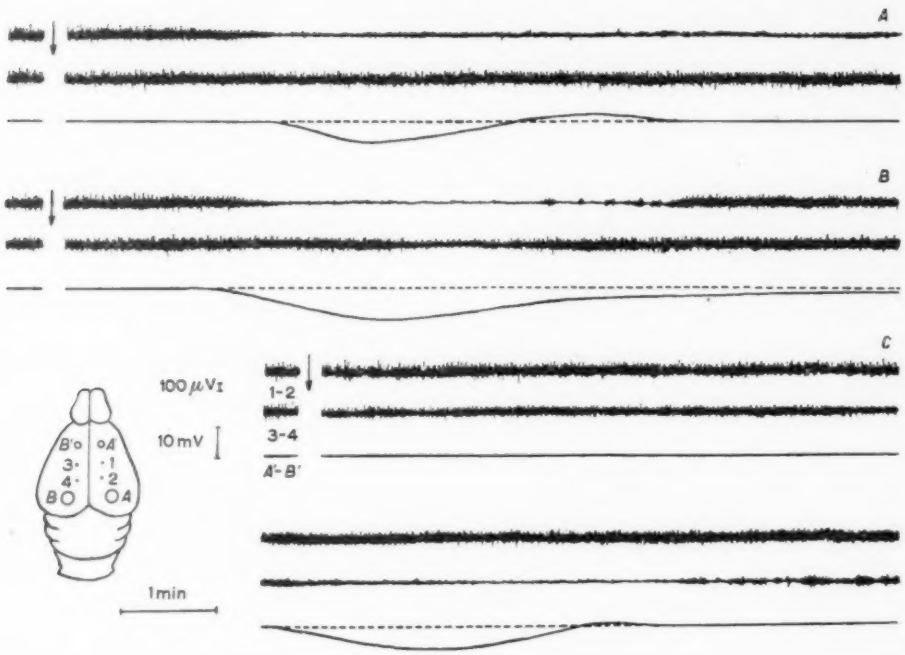


FIG. 2.—Spreading depression evoked by various inhibitors.
The arrow indicates the application of 2,4-dinitrophenol in record *A*, of NaCN in *B*, and of NaF in *C*. Spreading depression was elicited from trephine opening *A* in records *A* and *B*, from trephine opening *B* in record *C*. The downward deflection of the slow potential change record denotes increased negativity of the hemisphere, in which spreading depression was elicited in all experiments.

TABLE 4.—THE MEAN THRESHOLD CONCENTRATIONS FOR METABOLIC INHIBITORS

	ED 50 (M)	log ED 50 ± SD
2,4-DNP	$2{\cdot}08 \times 10^{-4}$	$-3{\cdot}682 \pm 0{\cdot}263$
NaCN	$1{\cdot}51 \times 10^{-3}$	$-2{\cdot}820 \pm 0{\cdot}093$
NaF	$1{\cdot}05 \times 10^{-2}$	$-1{\cdot}980 \pm 0{\cdot}088$
NaN_3	$2{\cdot}50 \times 10^{-2}$	$-1{\cdot}609 \pm 0{\cdot}079$

these substances. These results show the important role of carbohydrate metabolism and oxidative phosphorylation in the initiation of spreading depression.

DISCUSSION

In the experiments described above, a striking analogy was found between some metabolic reactions of brain slices *in vitro* and spreading depression in the cerebral cortex *in vivo*. It is possible that increased Q_{O_2}, accumulation of lactic acid and decrease of ATP are normal concomitants of spreading depression. Such an assumption is in good agreement with the paper of VAN HARREVELD and STAMM (1952) who observed a steep fall of cortical pO_2 corresponding to the steady potential

change in spreading depression. According to BUREŠOVÁ (1956) this pO_2 decrease cannot be caused by vasoconstriction, as VAN HARREVELD and STAMM originally suggested, because by more direct methods (haemoglobin determination and use of cooled thermocouples) only vasodilatation was observed to accompany the spreading depression. With brain circulation and arterial-blood O_2 saturation undiminished, the pO_2 decrease can be explained only by increased O_2 consumption.

If this assumption is correct another question arises: are these metabolic features of spreading depression important for the spreading mechanism, or for the initiation of spreading depression? From our experiments with NaCN it seems improbable that Q_{O_2} increase is a necessary condition for the development of spreading depression. A feature which is common to all the substances used (potassium, glutamine, inhibitors, etc.) is a change in the level of "high-energy" phosphate. At present the question whether the decrease of $\sim P$ bonds observed in brain slices is due to greater energy utilization or to the slower formation of these compounds remains unanswered. Similarly it remains unknown whether spreading depression is a passive or an active reaction, whether it may be considered as a transient collapse of cortical polarity or, on the contrary, as certain kind of neuronal hyperactivity.

From our results it is concluded that spreading depression can be elicited by a reaction in which the carbohydrate metabolism is involved. Different stimuli—electrical, mechanical, and chemical—probably lead to some specific metabolic reaction, similar perhaps to that in brain slices, which has the ability to start a process travelling at a very slow rate through the cortex. It must be stressed, however, that our findings concern only the process which initiates the spreading depression, and not the spreading mechanism itself. Further data are required therefore to extend this analogy or to demonstrate it by chemical methods.

SUMMARY

Local application of filter papers saturated with KCl solutions to the cortical surface elicits a well-defined ECoG reaction, spreading depression, in anaesthetized rats. Substances eliciting similar metabolic reactions in brain slices—KCl, NH_4Cl, glutamine, 2,4-dinitrophenol, veratrine—evoke spreading depression when applied locally to the cortex. Substances blocking the metabolic effect of potassium in brain slices (citrate, α-ketoglutarate, succinate, malonate) prevent the initiation of spreading depression when added to effective KCl solutions *in vivo*. There is thus a certain analogy between the effect of potassium on brain slices and the mechanism by which potassium gives rise to spreading depression. It is also possible to produce spreading depression by NaN_3, NaCN, and NaF.

Acknowledgements—The author is indebted to Prof. CH. S. KOSHTOYANS of Moscow University, who suggested this subject for investigation. The author acknowledges with thanks the excellent technical assistance of Miss V. KAISEROVÁ and Miss S. HERMANOVÁ and the valuable advice and constructive criticism of Dr. I. RYCHLÍK from the Chemical Institute of the Czechoslovak Academy of Sciences.

REFERENCES

BUREŠ J. and BUREŠOVÁ O. (1956) *Physiol. Bohemoslov.* **5**, 195.
BUREŠ J. and KOŠTOJANC CH. S. (1955) *DAN SSSR* **105**, 1118.
BUREŠOVÁ O. (1956) *Physiol. Bohemoslov.* In the press.

DIXON K. C. (1949) *J. Physiol.* **110,** 87.
ELLIOTT K. A. C. (1955) *Canad. J. Biochem. Physiol.* **33,** 466.
FINNEY D. J. (1950) *Probit Analysis,* Cambridge University Press, London.
GHOSH J. J. and QUASTEL J. H. (1954) *Nature,* **174,** 28.
GOLDRING S. and O'LEARY J. L. (1951) *J. Neurophysiol.* **14,** 275.
KIMURA Y. and NIWA T. (1953) *Nature,* **171,** 881.
LEÃO A. A. P. (1947) *J. Neurophysiol.* **10,** 409.
LEÃO A. A. P. and MORISON R. W. (1945) *J. Neurophysiol.* **8,** 33.
LIPSETT M. N. and CRESCITELLI F. (1950) *Arch. Biochem.* **28,** 329.
MCILWAIN H. (1954) *EEG Clin. Neurophysiol.* **6,** 93.
VAN HARREVELD A. and STAMM J. S. (1952) *J. Neurophysiol.* **15,** 487.
VAN HARREVELD A. and STAMM J. S. (1953) *Amer. J. Physiol.* **173, 164.**
WEIL C. S. (1952) *Biometrics* **8,** 249.
WEIL-MALHERBE H. (1938) *Biochem. J.* **32,** 2257.
WOLLENBERGER A. (1955) *Biochem. J.* **61,** 68.

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 159–165, Pergamon Press Ltd., London.

THE CONTENT AND THE CONCENTRATION OF RIBONUCLEIC ACID IN MOTOR ANTERIOR HORN CELLS FROM THE RABBIT

J.-E. EDSTRÖM
Department of Histology, University of Gothenburg, Sweden

(*Received* 19 *September* 1956)

THE basophilic material of the nerve cell has been the object of a large number of investigations. The striking alterations found in it in various physiological and pathological states have attracted much attention and led to much speculation about its function (for literature, see HYDÉN, 1943). The following facts make it likely that the basophilic and ultra-violet-absorbing component is ribonucleic acid (RNA): (a) the affinity for basic stains has been shown to be a property of nucleic acids in *in vitro* experiments (MICHAELIS, 1947); (b) ultra-violet microphotometry has shown it to have an absorption curve practically identical with that of RNA (HYDÉN *et al.*, 1941): (c) it is extracted by ribonuclease (BRACHET, 1940; GERSH and BODIAN, 1943); and (d) finally it has been found to contain the four mononucleotides characteristic of RNA (EDSTRÖM and HYDÉN, 1954; EDSTRÖM, 1956).

The motor anterior horn cells belong to the neurons rich in basophilic material. Preliminary estimations have been made of the amount and concentration of RNA in these cells in the rabbit (EDSTRÖM, 1953a,b). In the earlier of the reports cited, where freshly fixed cells were used, they were found to contain about 220 pg of RNA (1 pg = 10^{-12} g) and to have a mean concentration of about 0·7% (w/v). In the latter report, 550 pg and 2·4% are the values given for cells first freeze-dried and afterwards fixed. The discrepancy between the values obtained after the two different treatments is evident and calls for a reinvestigation.

A further object of the present investigation was to obtain a picture of the distribution of RNA content and volume of the nerve cell body. It is well known that the mean value for the cell volume varies at different segmental levels in the spinal cord. The question is whether this applies also to RNA. It has been suggested that there may be a correlation between the amount of basophilic substance and the plasma volume (HEIDENHEIN, 1911). The present study was undertaken partly with the object of investigating whether there is any relation between the amount of RNA in a nerve cell body and its volume or some other function of the cell body.

MATERIALS AND METHODS

Young rabbits of 1·5–2 kg were used. The animals were killed by decapitation, the spinal canal was opened, and the cervical cord removed. It was cut into 1 mm-thick pieces at the thickest part of the cervical enlargement; they were then either fixed for an hour in CARNOY's solution (ethanol, chloroform, conc. acetic acid, 6 : 3 : 1, by volume) or placed in isopentane cooled with liquid air. The frozen pieces were then dried at $10^{-2\cdot3}$ mm Hg and −40°C for a week, after which they were fixed for an hour in CARNOY's solution. Fixed pieces were transferred *via* ether to benzol and embedded in paraffin.

The majority of the cells (90) were taken from one rabbit, where about half the number (50) were from freeze-dried sections and the rest (40) from freshly fixed material. A second rabbit was used for analyses of 12 freeze-dried cells. These values have been published elsewhere (EDSTRÖM 1953b). Freshly fixed cells (15) were taken from a third rabbit. All cells except 10 freeze-dried ones from the first rabbit were taken from the anteriolateral nucleus.

Cell-body volume, RNA content, and concentration were determined as previously described (EDSTRÖM, 1953b). According to this method, the nerve-cell bodies are dissected out from 70 μ-thick sections by micromanipulation in an oil chamber. The cell body volumes are then determined by three-dimensional optical reconstruction (but instead of taking the mean value of three volume determinations obtained by focusing every 4 μ as described earlier, one determination was made per cell with focusings spaced at 2·3 μ intervals in the majority of cases). The cells are then extracted with a buffered ribonuclease solution. The extracts are placed on a cellophane strip which serves as a cuvette for ultra-violet microphotometry at 257 mμ, by which means values for the RNA content of the individual cell are obtained. By using the values for the cell body volume, concentration values are calculated.

RESULTS

Comparison between freeze-dried-fixed and freshly fixed material

RNA values plotted against cell body volumes for freeze-dried-fixed and freshly fixed cells respectively are shown in Fig. 1. It can be seen that the distribution is similar in both cases. There is no indication that freshly fixed material differs to any significant extent from freeze-dried-fixed material with regard to RNA content and cell body volume. The mean values for the freeze-dried-fixed cells lie around 530 pg of RNA, a volume of about 22×10^3 μ^3 and an RNA concentration of about 2·5% (w/v). The corresponding values for the freshly fixed cells are: 545 pg, 22×10^3 μ^3 and 2·6%.

Topographical distribution of values

Cells lying in the same section were often found to give values lying close to each other but differing markedly from those of cells in sections taken at some distance away. This applies to the volume of the cell body as well as to the RNA content and concentration. These results are illustrated in Fig. 2, where RNA concentration values for cells from one of the rabbits have been arranged according to the section examined.

Relation between RNA content and cell body volume

In Fig. 3 values for the RNA content of all the cells investigated have been plotted against the cell body volumes. It is evident that no direct proportionality exists. On the other hand, the values seem to be distributed around a line which gives the relation between the RNA amounts and the square of the third root of the volume. For individual measurements the correlation is not very close. If, however, the RNA content is proportional to the surface of the cell body, this is what is to be expected, since this function of the cell body volume gives only a mean value for the relationship between volume and surface in an irregular body like the motor anterior horn cell.

It therefore appeared to be of interest to see whether all the values fall within limits compatible with the hypothesis that the RNA content is proportional to the cell body surface. Assuming that the average shape of a motor anterior horn cell is a tetrahedron, cells which show a minimal surface-to-volume ratio, i.e. which are spherical, should lie on the lower of the lines drawn in Fig. 3. This line should

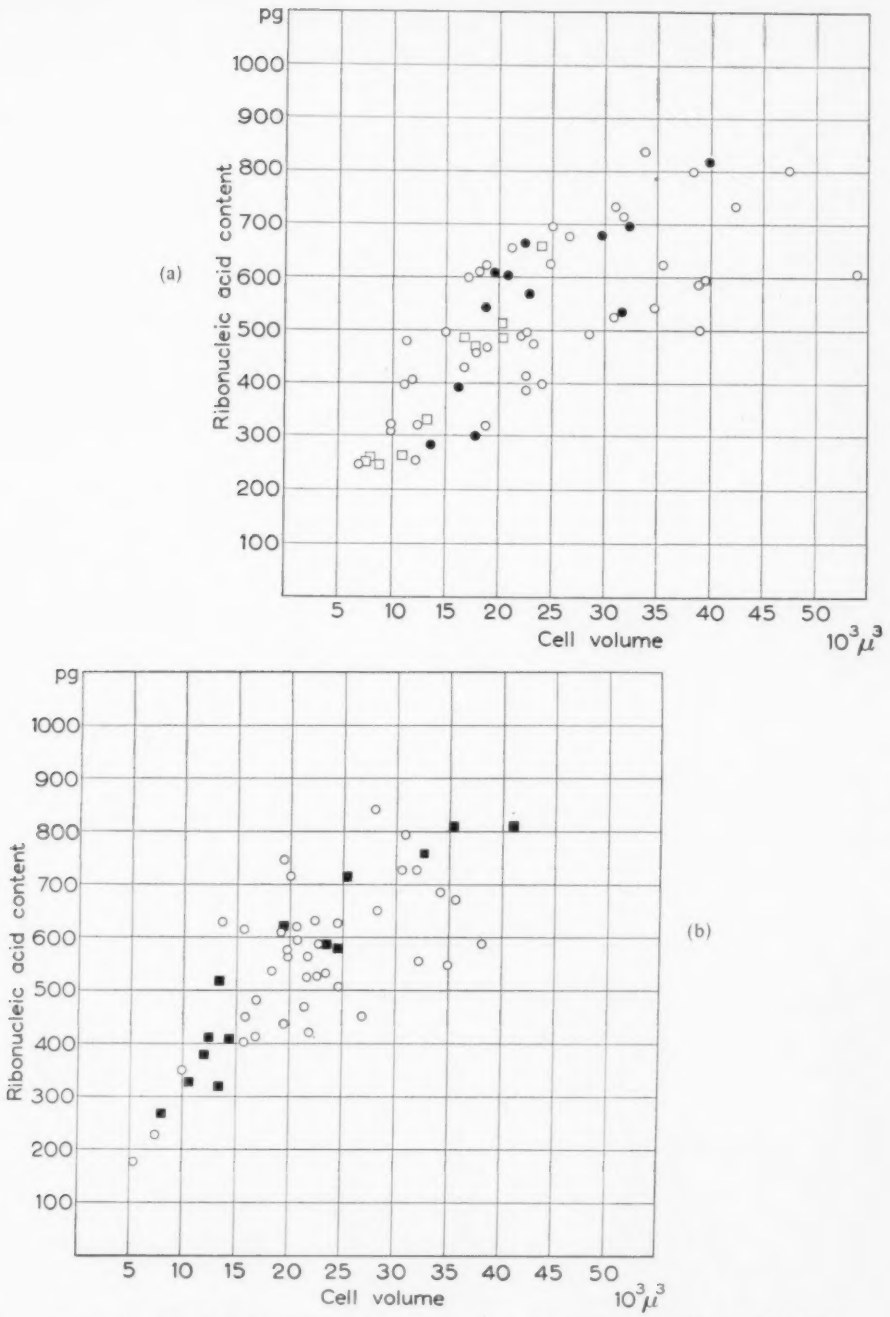


FIG. 1.—Values for RNA content plotted against cell body volumes for freeze-dried-fixed cells (a) and freshly fixed cells (b). Open circles denote cells from the anteriolateral nucleus of one animal. Open squares denote cells lying anteriomedially and dorsolaterally in the spinal cord of the same animal. Filled circles and filled squares show values from the anteriolateral nucleus of other rabbits.

VOL. 1 56/57

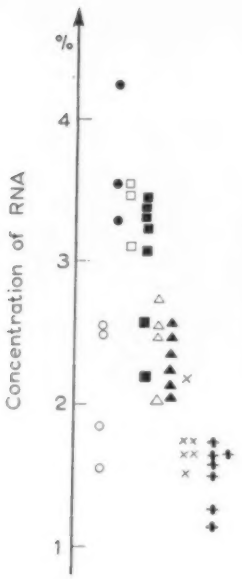


FIG. 2.—Illustration of the observation that RNA values for cells lying at the same level in the spinal cord are often similar. Values for cells from the same section have been given identical symbols. Freeze-dried-fixed cells from the anteriolateral nucleus have been used. The abscissae represents different sections.

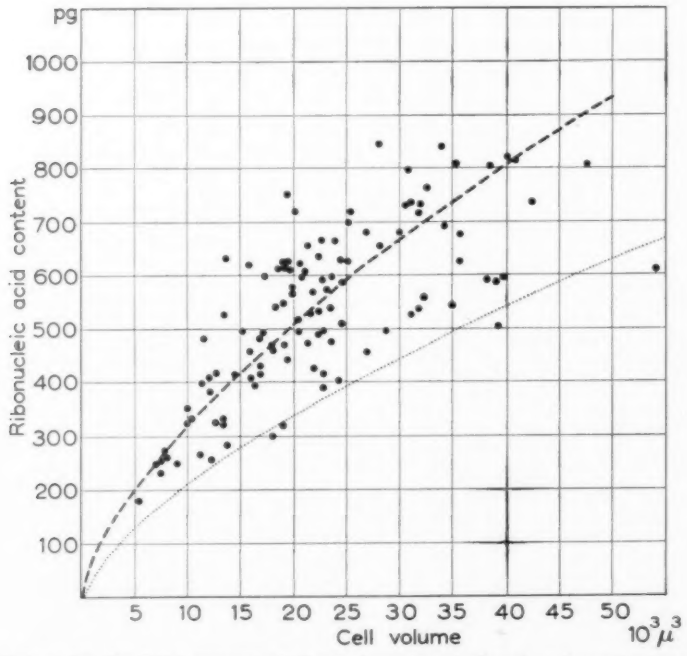


FIG. 3.—Values for the RNA content of all cells investigated plotted against cell volumes. The broken curve represents the mean correlation between RNA content and the square of the cube root of the volume. The dotted line represents the same correlation for spherical bodies under the assumption that the average shape of the cell bodies investigated is the regular tetrahedron. (In this figure, as in Fig. 1, two values fall outside the diagram, 1050 pg/59·5 $\times$ 10^3 μ^3, freeze-dried, and 1182 pg/37·4 $\times$ 10^3 μ^3, freshly fixed.)

VOL
1
1956/

also represent a limit within (above and to the left of) which the observed values should lie. It can be seen that this is actually the case. There is, of course, no theoretical upper limit for the surface-to-volume ratio. However, the values found fall within a sector showing a clear upper limit, the significance of which is unknown.

It is of interest to note that the mean value for about 120 normal hypoglossal nucleus cells from the rabbit, 200 pg at $5{\cdot}6 \times 10^3\ \mu^3$, lies near the mean curve (BRATTGÅRD *et al.*, 1956). Values for spinal ganglion cells from the rabbit, from an earlier publication (EDSTRÖM, 1953b), fall with one exception in the vicinity of the curve for round cells.

On the assumption that the curve representing mean values for the correlation of RNA amounts and cell body volumes corresponds to a regular tetrahedron, the amount of RNA per surface unit for these nerve cell bodies can be calculated. It amounts to $0{\cdot}095\ \text{pg}/\mu^2$.

DISCUSSION

The data obtained here indicate that there is no significant difference between the results of analyses on freshly fixed material and those on freeze-dried-fixed material. It has been shown that no losses of RNA occur from the cell bodies when the latter type of material is used (EDSTRÖM, 1953b). This then must also be valid for material fixed in a fresh state in the way done here: 1 mm-thick pieces and fixation for an hour. The same conclusions were arrived at by NURNBERGER *et al.* (1952), using different methods.

Values for the RNA concentration in motor anterior horn cells have previously been obtained by means of ultra-violet microspectrophotometry. HYDÉN *et al.* (1941) found 2–3% of RNA in cells from the rabbit after freeze-drying, HYDÉN and HARTELIUS (1947) 1·7% in cells from the rabbit after Carnoy fixation, NURNBERGER *et al.* (1952) 1·7% after Carnoy fixation as well as after freeze-drying in cells from the cat. It is seen that these values agree quite well with those obtained here.

The results obtained in this study make it evident that the values given earlier for freshly fixed material (EDSTRÖM, 1953a) are erroneous. The uncontrolled fixation seems to be the cause of the low values found. Data given by SYLVÉN (1951) indicate that Carnoy's solution may dissolve and extract considerable amounts of RNA when long fixation times, necessary for large tissue pieces, are employed.

It is apparent even from the examination of stained sections of the spinal cord that the neurons within a single nucleus, like the ventrolateral which was mainly used here, are arranged in groups. The difference in size at different levels is evident also at the cervical and lumbar enlargements. The study carried out here confirms the impression obtained from histological studies, not only with respect to cell-body volume, but also in showing the same variation to be true for the total content and mean concentration of RNA. It is known that neurons in the spinal cord supplying the same muscles are arranged together. It seems likely then that they are characterized by similar values for cell body volume, RNA content and concentration. An example of this is seen in the neurons belonging to the hypoglossal nucleus supplying the tongue muscles. They have cell body volumes in the range $(3\text{–}8) \times 10^3\ \mu^3$, RNA amounts around 200 pg, and a mean concentration around 3·5% (BRATTGÅRD *et al.*, 1956).

The distribution of neurons in groups implies considerable difficulties when data for RNA content or cell body volume are collected and mean values for an animal are desired. Data obtained even from the study of a large number of thin sections are of limited value when the sections are adjoining. Unless it is stated that measurements include cells widely spaced from each other, such data must be evaluated with these facts in mind (of course the use of a large number of experimental animals will eliminate this variation between groups). Since the variation between groups is large, any method designed for use on this material should be one that allows a large number of analyses. The random error in such a method need not be low.

It is clearly tempting to draw the conclusion that RNA content and cell surface are proportional in a cell like the neuron, the functional activity of which is expressed at the surface. Such a relation would imply that RNA is used for the specific function of the neuron. HYDÉN and co-workers (e.g. HYDÉN, 1943; HAMBERGER and HYDÉN, 1949) have shown that RNA and proteins are synthesized during functional activity. A proportionality between cell-surface and RNA content would then indicate that the physicochemical events at the membrane of the cell body in some way determine the formation of RNA. RNA, in its turn, in all likelihood controls the synthesis of proteins. The rate of RNA and protein synthesis should, according to this hypothesis, be proportional to the membrane area of the cell body at a given activity level.

VOL 1 1956/

SUMMARY

Values for cell body volumes, RNA content, and concentration in motor anterior horn cells of the rabbit have been obtained by the use of microextraction techniques and optical volume reconstruction. About 120 cells were investigated, half of them were freshly fixed, the rest were first freeze-dried and fixed afterwards. The results show that:

(1) There is no evident difference between the values for freeze-dried-fixed and freshly fixed cells.

(2) The volume values of the cell bodies are distributed around 22,000 μ^3, the RNA contents around 540 pg, and the concentration values around 2·5%.

(3) The figures given earlier (EDSTRÖM, 1953a) for the RNA content and concentration in freshly fixed cells have been found to be too low.

(4) The cells are arranged in groups in the ventrolateral nucleus of the spinal cord with respect to their values for cell body volume, RNA content, and concentration.

(5) The collected data indicate that in these neurons there is a proportionality between the surface of the nerve cell body and its content of RNA.

REFERENCES

BRACHET J. (1949) *Compt. rend. soc. biol.* **133,** 88.

BRATTGÅRD S.-O., EDSTRÖM J.-E., and HYDÉN H. (1956) unpublished results.

EDSTRÖM J.-E. (1953a) *Biochim. Biophys. Acta* **11,** 300.

EDSTRÖM J.-E. (1953b) *ibid.* **12,** 361.

EDSTRÖM J.-E. and HYDÉN H. (1954) *Nature* **174,** 128.

GERSH I. and BODIAN D. (1943) *J. Cell. Comp. Physiol.* **21,** 253.

HAMBERGER C.-A. and HYDÉN H. (1949) *Acta Oto-Laryng.* Suppl. 75, 82.

HEIDENHEIN M. (1911) in v. Bardeleben *Handbuch der Anatomie des Menschen*, Band VIII, 2 Lief. Plasma und Zelle.

HYDÉN H. (1943) *Acta Physiol. Scand.* Suppl. 17.
HYDÉN H. and HARTELIUS H. (1947) *Acta Psychiat. et Neurol.* Suppl. 48.
HYDÉN H., CASPERSSON T., and WOHLFAHRT G. (1941) *Ztschr. f. mikr.-anat. Forsch.* **49,** 534.
MICHAELIS L. (1947) *Cold Spring Harbor Symp. Quant. Biol.* 12, 131.
NURNBERGER J., ENGSTRÖM A., and LINDSTRÖM B. (1952) *J. Cell. Comp. Physiol.* **39,** 215.
SYLVÉN B. (1951) *Report on the Symposium of Freezing and Drying*, Inst. Biol., 189.

VOL.
1
56/57

Journal of Neurochemistry, 1956, Vol. 1, pp. 166–172, Pergamon Press Ltd., London.

ENZYMIC DEVELOPMENT AND MATURATION OF THE HYPOTHALAMUS

P. COHN* and D. RICHTER
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff (Great Britain)

(*Received* 25 *July* 1956)

VOL
1
1956/

FLEXNER (1955) has shown that the cytological changes occurring during the development of nervous tissues are associated with concurrent changes in the pattern of enzymic activity. Changes have been reported in the activity of various enzymes in a number of localized regions of the brain (NACHMANSOHN, 1939, 1940; YOUNGSTROM, 1941; HIMWICH and APRISON, 1955), but little systematic information is available, however, about the enzymes of the hypothalamus.

The hypothalamic centres are of interest in view of their role in relation to the chief autonomic and endocrine functions of the body, and since they may also influence the activity of the cerebral cortex (LE GROS CLARK and MEYER, 1950). The vegetative functions of the hypothalamus are particularly important in the early life of the animal. The development of biochemical activity in the hypothalamo-neurohypophysial system was studied by HELLER and ZAIMIS (1949), who found a lower concentration of antidiuretic hormone in the neurohypophysis of the newborn than of the adult rat. DAWSON (1953) observed the appearance of neurosecretory substance in the hypothalamus and posterior pituitary of the rat within a few days after birth.

In the present investigation the maturation of the hypothalamus of the rat was studied by following the changes with age in the activities of the enzymes phosphomonoesterase I and II (nonspecific alkaline and acid phosphatase) and acetylcholinesterase. The values obtained for the hypothalamus were compared with values obtained concurrently for the cerebral cortex and the thalamus. In addition, histochemical techniques were applied to study the localization of the two phosphomonoesterases in the different hypothalamic nuclei. More information was thus obtained about the distribution of these enzymes than could be obtained by biochemical or histochemical methods alone.

EXPERIMENTAL

Material. A total of 151 animals from twenty-eight litters of Wistar albino rats were assigned at birth to be used at 1, 3, 7, 10, 15, 25, 40, 60, and 100 days of age for the estimation of enzymes in the brain. The young rats from each litter were kept together in the same cage even after they had been weaned.

Each rat was killed by decapitation, the brain carefully dissected out, and superficial blood removed with the aid of moistened hard filter paper. After the brain had been weighed it was put in a Petri dish lined with moistened filter paper and

* Present address: Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London S.W.3.

frozen to a wax-like consistency at −5°. A sample of cerebral cortex was then shaved off with a sharp scalpel and rapidly weighed on a torsion balance. The hypothalamus was defined by making incisions 2 mm deep just rostrally to the optic chiasma, caudally to the mammillary bodies, and along the lateral borders of the tuber cinereum. This block of tissue was carefully severed from the main portion of the brain by passing the scalpel horizontally in a plane just ventral to the anterior commissure. The accuracy of this process of dissection was checked by histological examination of the tissue thus removed. Its weight was found to increase with age from approximately 15 mg at birth to nearly 80 mg in the fully grown rat. Finally, samples of the thalamus were taken. The parts from the brains of each age-group were divided into 2–4 sets of pooled samples for separate estimation.

Suspensions were prepared with distilled water to contain 91 mg fresh tissue/ml. The dry weights were estimated by drying 2 ml to constant weight at 105°.

Enzyme activities. (i) Phosphomonoesterase estimations were carried out within two hours of preparation of the suspension by the method of KING and ARMSTRONG (1934). The enzymic hydrolysis of disodium phenylphosphate by phosphomonoesterase I was carried out at pH 9·8 for 15 min in the presence of magnesium chloride (10^{-3} M). The action of phosphomonoesterase II on this substrate was allowed to proceed at pH 4·9 for 60 min. The density of the blue colour given by the liberated phenol on addition of the reagent of FOLIN and CIOCALTEU was determined in a photoelectric absorptiometer. The activities were expressed in King-Armstrong units; mg phenol liberated at 37° under standard conditions by 1 g. of dry tissue.

(ii) The acetylcholinesterase was determined manometrically in the Warburg apparatus. Acetyl-β-methylcholine chloride (final concentration 0·03 M) was used as substrate. It was dissolved in 0·025 M-sodium bicarbonate before being placed in the side arm of a Warburg flask. Sodium bicarbonate was included in the total volume (1·60 ml) of liquid to give a concentration of 0·025 M. The flasks were filled with CO_2 5%—N_2 95% at 37·4°. Enzyme activities were calculated from the rate of evolution of carbon dioxide after deduction of that due to nonenzymic hydrolysis. The enzyme activities of the tissue preparations were expressed as μM substrate hydrolysed by 1 g. of dry weight per hour.

Histochemical investigations

(i) *Phosphomonoesterase I activity.* The brain was cut transversely into slices 2–3 mm thick. For the examination of phosphomonoesterase I activity these slices were fixed in ice-cold 90% ethanol, dehydrated in absolute ethanol, cleared in cedarwood oil and embedded in paraffin. Sections 7–8 μ thick were incubated at 37° and treated subsequently according to techniques described by DANIELLI (1946, 1950) and MARTIN and JACOBY (1949). The solution used for incubation contained disodium phenylphosphate (0·005 M) as substrate together with calcium nitrate (0·013 M) and magnesium sulphate (0·01 M) buffered with sodium barbitone (0·067 M). In control experiments the substrate was omitted.

(ii) *Phosphomonoesterase II activity.* Fresh-frozen sections of unfixed tissue were cut for the localization of phospomonoesterase II activity. The composition of the incubation medium and the procedure of washing and fixing the preparation were those described by NAIDOO and PRATT (1951). The sections were then dehydrated, cleared, and mounted for examination.

RESULTS

Biochemical estimations

Fig. 1 shows the changes in phosphomonoesterase I (alkaline phosphatase) activity in the hypothalamus, thalamus, and cerebral cortex of the rat brain from birth to adult life. In the brain of the 1-day-old rat the hypothalamus and the cerebral cortex

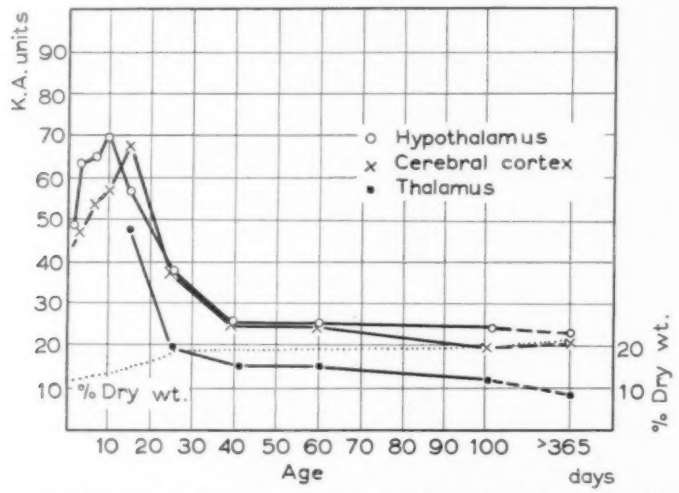


FIG. 1.—Phosphomonoesterase I activity in the hypothalamus, cerebral cortex, and thalamus of the rat during growth.

were found to possess phosphomonoesterase I activities twice those of the adult animal. The enzyme was observed to reach its highest activity in the hypothalamus at the age of 10 days, and in the cerebral cortex by the fifteenth day of life. The greatest scatter was observed in the values obtained up to about 15 days (Table 1).

TABLE 1.—THE DEVELOPMENT OF ENZYMIC ACTIVITY IN THE HYPOTHALAMUS AND CEREBRAL CORTEX OF THE RAT BRAIN

(For animals aged 3–40 days each figure is the mean of duplicate determinations on four sets of pooled samples. For animals aged 60 days three sets of pooled samples were used. All values represent enzyme units ± standard error of the mean.)

Age (days)	Number of rats	Hypothalamus			Cerebral cortex		
		Phosph. I	Phosph. II	Acetyl-cholin-esterase	Phosph. I	Phosph. II	Acetyl-cholin-esterase
3	21	63 ± 7	26 ± 5	740 ± 84	47 ± 2	22 ± 2	188 ± 6
7	20	65 ± 6	31 ± 2	870 ± 25	54 ± 3	30 ± 4	333 ± 44
10	16	70 ± 6	33 ± 3	1000 ± 113	57 ± 6	28 ± 4	321 ± 76
15	15	57 ± 5	31 ± 3	1230 ± 81	68 ± 12	29 ± 3	385 ± 40
25	16	38 ± 3	27 ± 3	1060 ± 99	38 ± 3	24 ± 2	435 ± 21
40	11	25 ± 1	22 ± 3	1020 ± 55	25 ± 1	22 ± 2	467 ± 88
60	7	24 ± 1	25 ± 1	1080 ± 60	25 ± 1	24 ± 2	608 ± 43

The values for the thalamus were found to be lower than those for the other two parts throughout the period of growth examined. Phosphomonoesterase I activities in the hypothalamus, cerebral cortex, and thalamus fell from their maximum to the levels present in the adult between the fortieth and the sixtieth day.

The relation to age of the levels of phosphomonoesterase II activity was similar to

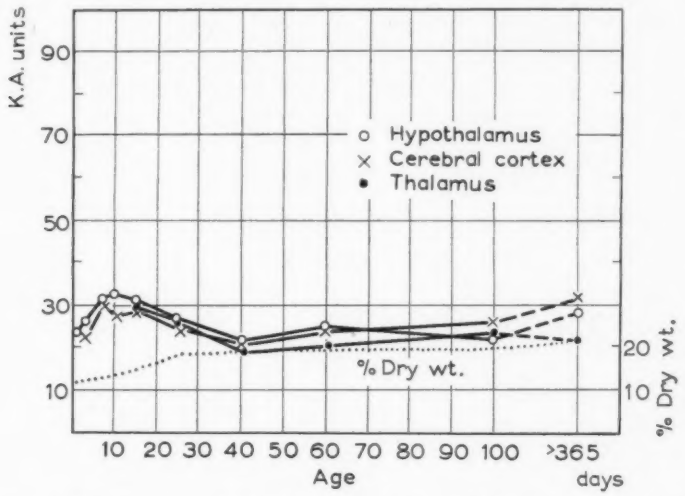


FIG. 2.—Phosphomonoesterase II (acid phosphatase) activity in the hypothalamus, cerebral cortex, and thalamus of the rat during growth.

that observed for phosphomonoesterase I, though the quantitative changes were less pronounced (Fig. 2). Maximum activity occurred during the period 7 to 15 days after birth. In the hypothalamus, cerebral cortex, and also in the thalamus, enzyme activity decreased until the fortieth day of age, when a similar level was reached in all three parts. In the hypothalamus and cerebral cortex of rats over one year old higher values were observed than at the age of 100 days.

Acetylcholinesterase activity in the hypothalamus was found to increase rapidly from the first day of life to the fifteenth (Fig. 3), after which the values dropped slightly and then remained practically constant until later in life, when there was some indication of a further fall in activity. On the other hand, the acetylcholinesterase activity in the cerebral cortex was highest at about the sixtieth day. These observations were made with acetyl-β-methylcholine as substrate, and further experiments with acetylcholine as substrate gave a closely similar picture. Experiments with benzoylcholine showed that under these conditions the activity of the butyrylcholinesterase in the hypothalamus and cerebral cortex was relatively small.

Histochemical investigations

Sections from rats belonging to eleven different litters were treated and examined for phosphomonoesterase I and II activities. At least two rats were drawn from each group. In the brain of the new-born rat many capillaries, and particularly those of the choroid plexus, possessed a higher phopshomonoesterase I activity than nervous

tissue. The activity was fairly even among various regions of the brain; however, structures around the third ventricle and the middle layers of the cerebral cortex were more deeply stained than many other parts (Fig. 4). Several preparations from 7-day-old rats showed a supraoptic nucleus which could easily be detected by its prominent activity. A common feature of the sections of brains from young rats was well-marked staining of the fibre tracts such as the optic chiasma and the corpus callosum. A close examination of individual neurones showed the sparse and agranular cytoplasm and the nucleolus to be much more deeply stained than the nucleus.

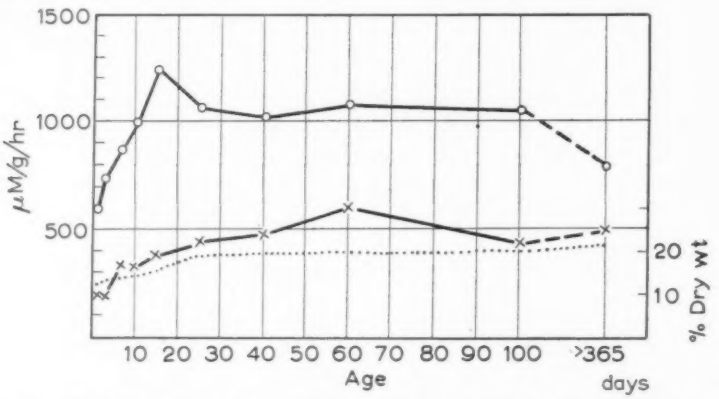


FIG. 3.—Variation in acetylcholinesterase activity with age in the hypothalamus (○) and cerebral cortex (×) of the rat.

Phosphomonoesterase II activity in the brain of the very young rat could easily be detected in the ependyma of the choroid plexus. Most other parts of the brain were only feebly active. By the tenth day several hypothalamic nuclei could be made out by their being stained more deeply than most of the basal ganglia and the cerebral cortex. The 15-day-old rat possessed high phosphomonoesterase II activity in such nuclei as the supraoptic, paraventricular, and suprachiasmatic. Phosphomonoesterase II was demonstrated after longer periods of incubation in parts of the hippocampus, the ependymal lining of the third ventricle, and the dorsomedial, ventromedial, and lateral mammillary nuclei (Fig. 5). Little differentiation among the layers of the cerebral cortex was visible; in general, these appeared much paler than regions in the brain stem. At the cytological level, the nucleolus of the cell exhibited the deepest staining, the cytoplasm was seen to be paler and the cell nucleus even more so.

Between the fifteenth and sixtieth day phosphomonoesterase activities tended to assume the adult levels and patterns of distribution. In the cerebral cortex, the staining for phosphomonoesterase I throughout the different layers became more even. Fibre tracts decreased in activity when compared with adjacent regions of grey matter.

DISCUSSION

From the foregoing results two main patterns of enzymic development in the hypothalamus and in the cerebral cortex can be made out. The activities of both phosphomonoesterases rise rapidly from their levels at birth to maxima between the tenth and fifteenth days of life, and fall again. In contrast, acetylcholinesterase activity increases from the value found in the new-born to that in the young adult rat.

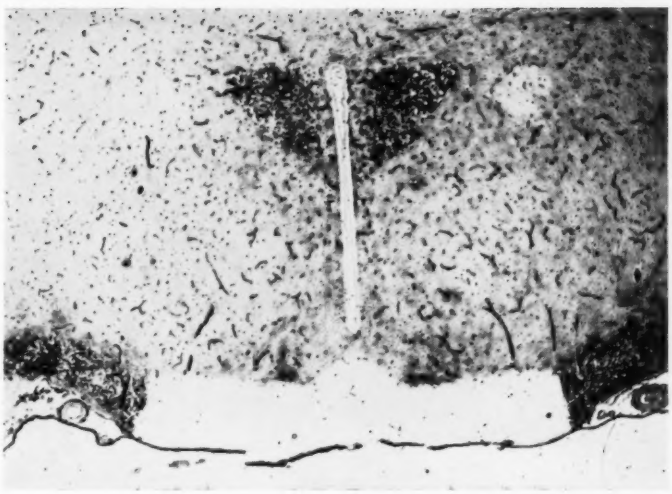


FIG. 4.
Alkaline phosphatase activity in the hypothalamus of the adult rat (×30). The capillary network and many of the neurones of the supraoptic, suprachiasmatic and paraventricular nuclei are deeply stained.

VOL.
1
56/57

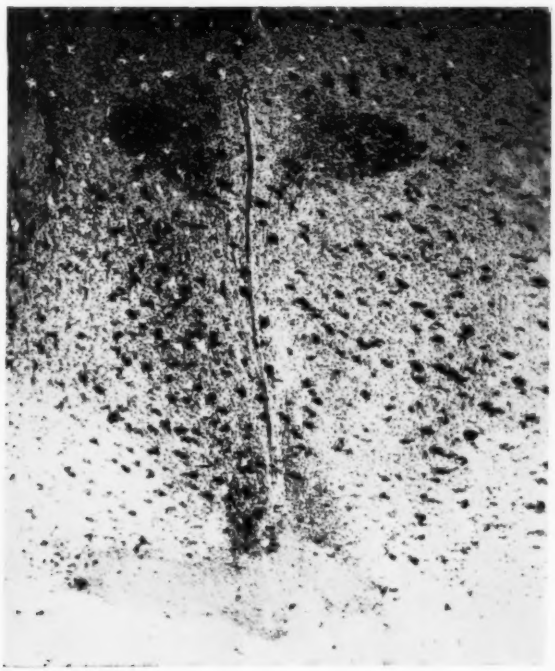


FIG. 5.
Acid phosphatase activity in the hypothalamus of the adult rat (×40). Activity in the paraventricular and suprachiasmatic nuclei.

facing p. 170

VOL
1
1956/

It is, therefore, conceivable that the activities of these two groups of enzymes change with age in response to, or as a result of, two different requirements governed by the stage of development.

The rapid rises in the activities of both phosphomonoesterases occur during a period of growth which has been observed by KOCH and KOCH (1913) to be characterized by an intense protein anabolism. The physiological roles of phosphomonoesterase I and II are still obscure. There is evidence, however, that both these enzymes are abundant at many sites of protein synthesis (MOOG, 1946; DAVIDSON, 1953). Support for the view that a relationship exists in the hypothalamus between phosphomonoesterases and protein formation was given by the histochemical and cytological studies of ERÄNKÖ (1951), who concluded that the neurosecretory cells of the supraoptic nucleus were a site of active protein synthesis. Direct biochemical experiments using an autoradiographic technique have shown (COHN, GAITONDE, and RICHTER, 1954) that ^{35}S-methionine is rapidly incorporated into the tissue proteins of the supraoptic nucleus, where phosphomonoesterases are now shown to be active soon after birth. These findings are of special interest in view of the cytological evidence (BARGMANN, 1954) that points to a synthesis in the hypothalamus of polypeptide hormones which are subsequently released by the neurohypophysis.

Despite the differing patterns of development of phosphomonoesterase I and acetylcholinesterase, both reached maximal activity in phylogenetic order in hypothalamus, thalamus and cortex. In the hypothalamus and the cerebral cortex, enzymic maturity of phosphomonoesterase I and II was found to precede that of acetylcholinesterase. Such a relation with age may be evidence of a simultaneous occurrence of incremental growth and chemical differentiation. The findings of FLEXNER and his collaborators of a constant protein concentration (FLEXNER and FLEXNER, 1950) but of a rise in the activities of adenosine triphosphatase (FLEXNER and FLEXNER, 1948), succinic dehydrogenase and cytochrome oxidase (FLEXNER, 1952) in the cerebral cortex of the guinea-pig from the "critical period" onwards (41 to 45 days foetal age; 8 to 10 days after birth in the rat), showed that from that time differentiation of the protein into enzymes began to supersede incremental growth. Similarly, for suspensions of whole rat brain, POTTER, SCHNEIDER, and LIEBL (1945) reported a more rapid increase in the activities of succinic dehydrogenase, cytochrome oxidase, and adenosine triphosphatase than in the dry weight of the tissue between the fifth and twenty-fifth day of life. BAYLISS and TODRICK (1953) observed that acetylcholinesterase in whole brain suspensions had reached its adult level of activity by the twenty-fifth day of life. In comparison, it should be noted that the present examination of well circumscribed parts of the brain revealed an earlier maturation in the hypothalamus and a much later maturation in the cerebral cortex than that reported by BAYLISS and TODRICK for whole brain suspension. The later maturation of the cerebral cortex was indicated also by the results of ELKES and TODRICK (1955). In this connection it is of interest that the acetylcholine content of the rat brain at 100 days of age has been found to be twice that at 21 days (CROSSLAND, 1951).

The application of biochemical methods of estimation and of histochemical techniques concurrently made it possible, in the present investigation, to obtain evidence of varying levels of phosphomonoesterase activities among the different hypothalamic nuclei during the period of development. The significance of the presence of the phosphomonoesterases in nervous tissue is still unknown. The predominantly

cytoplasmic site of activity of phosphomonoesterase II, however, should be contrasted with its relatively small increase in activity at the "critical period," when the cytoplasm becomes granular and cell processes grow rapidly.

SUMMARY

(1) The enzymes phosphomonoesterase I and II and acetylcholinesterase were estimated in the hypothalamus, the cerebral cortex, and the thalamus of growing and adult rats. Phosphomonoesterase I and II activity of the brain was also examined histochemically.

(2) Phosphomonoesterase I and II activities in the hypothalamus and the cerebral cortex rose from their levels at birth to a peak value between the tenth and fifteenth day, and subsequently fell again.

(3) Acetylcholinesterase activity reached maximum values in the hypothalamus at the fifteenth day, but in the cerebral cortex not until the sixtieth day of life.

(4) Phosphomonoesterase I and II was observed to be present in appreciable activity in certain of the hypothalamic nuclei of rats soon after birth.

Acknowledgements—The competent technical assistance given by Miss J. M. GILSON is gratefully acknowledged. One of the authors (P. C.) wishes to thank the Rockefeller Foundation for a maintenance grant and Mr. K. MOREMAN for help in the preparation of Fig. 3. This investigation formed part of a thesis approved by the University of London for the degree of Ph.D.

REFERENCES

BARGMANN W. (1954) *Publ. Staz. zool. Napoli* **24,** (Suppl.), 11.
BAYLISS B. J. and TODRICK A. (1953) *Biochem. J.* **54,** xxix.
CLARK W. E. LE GROS and MEYER M. (1950) *Brit. med. Bull.* **6,** 341.
COHN P., GAITONDE M. K., and RICHTER D. (1954) *J. Physiol* **126,** 7P.
CROSSLAND J. (1951) *J. Physiol.* **114,** 318.
DANIELLI J. F. (1946) *J. Exp. Biol.* **22,** 110.
DANIELLI J. F. (1950) *Nature* **165,** 762.
DAVIDSON J. N. (1953) *The Biochemistry of the Nucleic Acids.* Methuen & Co, London.
DAWSON A. B. (1953) *Anat. Rec.* **117,** 620.
ELKES J. and TODRICK A. (1955) *Biochemistry of the Developing Nervous System* Ed. H. WAELSCH, Academic Press, New York.
ERÄNKÖ O. (1951) *Acta. Med. exp. Fenn.* **29,** 158.
FLEXNER L. B. (1955) *Biochemistry of the Developing Nervous System* Ed. H. WAELSCH, Academic Press, New York.
FLEXNER J. B. and FLEXNER L. B. (1948) *J. cell. comp. Physiol.* **31,** 311.
FLEXNER J. B. and FLEXNER L. B. (1950) *Anat. Rec.* **106,** 413.
FLEXNER L. B. (1952) *Harvey Lect.* **47,** 156.
HELLER H. and ZAIMIS E. J. (1949) *J. Physiol.* **109,** 162.
HIMWICH H. E. and APRISON M. H. (1955) *Biochemistry of the Developing Nervous System.* Ed. H. WAELSCH, Academic Press, New York.
KING E. J. and ARMSTRONG A. R. (1934) *Canad. med. Ass. J.* **31,** 376.
KOCH W. and KOCH M. L. (1913) *J. Biol. Chem.* **15,** 423.
MARTIN B. F. and JACOBY F. (1949) *J. Anat. Lond.* **83,** 351.
MOOG F. (1946) *Biol. Rev.* **21,** 41.
NACHMANSOHN D. (1939) *Bull. Soc. Chim. biol. Paris.* **21,** 761.
NACHMANSOHN D. (1940) *J. Neurophysiol.* **3,** 396.
NAIDOO D. and PRATT O. E. (1951) *J. Neurol. Neurosurg. Psychiat.* **14,** 287.
POTTER V. R., SCHNEIDER W. C., and LIEBL G. J. (1945) *Cancer Res.* **5,** 21.
YOUNGSTROM K. A. (1941) *J. Neurophysiol.* **4,** 473.

VOL
1
1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 173–180, Pergamon Press Ltd., London.

QUANTITATIVE HISTOCHEMICAL CHANGES DURING THE DEVELOPMENT OF THE RAT CEREBRAL CORTEX*

ROBERT E. KUHLMAN† and OLIVER H. LOWRY
Department of Pharmacology, and the May-Beaumont Institute of Neurology,
Washington University School of Medicine, St. Louis, Missouri

(*Received* 1 *May* 1956)

VOL.
1
6/57

SUGITA (1917) described in detail the postnatal changes of the albino rat brain. He pointed out its immaturity and the lack of differentiation at birth when only four layers of cortex are present (Fig. 1). SUGITA divided the subsequent development into three phases: Phase I, starting at birth and lasting 10 days, is a period of rapid growth during which brain weight increases from 250 mg to 960 mg and the thickness of the cortex from 0·74 mm to 1·7 mm. During this phase cells migrate from the ventricles, multiply, and become organized, with the result that all fundamental cortical layers are present at 10 days of age, and all the major areas of the adult cortex are recognizable. Phase II extends from the 10th to the 20th day after birth. It is characterized by cellular enlargement and the development of axons, but few new cells make their appearance. Myelinization begins in the latter half of this phase. Brain weight increases from 960 mg to 2·0 g and cortical thickness increases to within 4 per cent of the final adult average. By the 20th day the cortex is completely organized. Phase III extends from the 20th to the 90th day and is the period in which myelinization is completed.

The rat brain is peculiar in that it continues to increase in weight throughout life; however, there is no appreciable increase in cortical thickness or cellular arrangement after the 20th day. Comparable physiological changes follow this anatomical development. Electrical activity recorded from the cerebral cortex is irregular and of low amplitude from 0 to 7 days of age, but by 10 days it is essentially similar to that in the adult (CRAIN, 1952). From a blind, poorly integrated, dependent animal at birth, the rat becomes, by 20 days, an active and autonomous unit.

Recently FLEXNER and his co-workers (1948, 1950, 1951, 1953) have investigated the biochemical changes during the "critical period" as it occurs (*in utero*) in the guinea-pig. They demonstrated rapid rises in ATPase, succinic dehydrogenase and cytochrome oxidase activity in whole brain homogenates. The levels of phosphocreatine and ATP were also studied throughout gestation. SUGITA (1917) in his investigation of the pre- and post-natal cellular changes of the rat cerebral cortex, found a rapid increase in the average cell and nuclear diameter of the pyramidal cells of layers III and V during the first 10 days after birth. These findings were similar to those of PETERS and FLEXNER (1950) for the guinea-pig. POTTER (1945) measured

* Supported in part by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.
† Washington University Student Research Fellow.

6 (20 pp.)

succinic dehydrogenase, cytochrome oxidase and ATPase activity in whole rat brain and noted relatively constant levels (*in utero*), with three- to six-fold increases from the 7th to 15th day after birth. These changes are greater than could be accounted for by changes in total solids, which increase from 12 per cent at birth to 13 per cent at 10 days and 22 per cent in the adult (DONALDSON and HATAI, 1911). Other studies by numerous authors have supported the conclusion that major changes occur in enzymatic patterns in the developing brain.

Most of these studies were made using the whole brain or the whole cortex. The composition of the brain obviously reflects (a) the composition of its histological components—cell bodies, processes, glia, myelin sheaths—and (b) the relative amounts of each of these histological elements. Analysis of the whole brain does not distinguish changes in one category from changes in the other.

In an attempt to make this distinction, the activities of four enzymes in individual cortical layers have been determined, using quantitative microchemical procedures applied to newborn, 10-day-old and adult rats. The enzymes selected for study were lactic dehydrogenase (LDH), malic dehydrogenase (MDH), glutamic dehydrogenase (GDH), and glucose-6-phosphate dehydrogenase (G6DH). These four were chosen with a view to assessing the relative importance of four of the major metabolic pathways or systems in the brain. In addition the total lipide and the concentration of total solids were measured. The histochemical data are supplemented with data from whole brain of rats at 0, 7, 11, 21 and 200 or more days of age.

VOL
1
1956/

MATERIALS AND METHODS

The brain tissue was obtained from rats of the Sprague-Dawley strain. The general procedure for obtaining suitable unfixed, unstained histologically defined material has been described elsewhere (LOWRY, 1953). The cortex was frozen in liquid nitrogen, sectioned (20 μ) at $-15°$ and dried at $-40°$. The tissue was sectioned obliquely to the surface of the cortex so that several cortical layers were present in each section. This made it relatively easy to identify each layer in the frozen-dried material, and yet it was possible to obtain larger samples of each cortical layer than would have been possible with cross sections. The cortex of newborn and 10-day-old rats was difficult to section because of the high water content and consequent tendency to fragment.

The histochemical studies, since they involved multiple estimations on a number of cortical layers, were performed only on a single rat of each age group. The results might therefore be characteristic of an individual rather than an age group. To avoid this difficulty in part, whole brains (above the pons) were removed from groups of rats of age 0, 7, 11, and 21 days and from adults (each litter provided one rat of each age and the parents served as adult controls), homogenized in 0·01 M trishydroxymethylaminomethane (TRIS) buffer (pH 7·5) and analysed for the same enzyme activities as the histological specimens.

The tissue fragments to be analysed weighed from 0·3 to 1 μg. The general procedures for weighing and for performing chemical analyses on the necessary micro scale have been given (LOWRY, 1953; LOWRY *et al.*, 1954 a, b). The lipid-free weight was measured after extraction with alcohol and hexane (LOWRY, 1953). The total solids (g. of total dry weight per litre) were measured by calculating the volume of a weighed sample from its area and thickness (LOWRY, 1953). It is believed that the values observed are somewhat high, owing possibly to shrinkage in drying. They are nevertheless recorded, since they may be of value in showing changes.

LDH and MDH were determined spectrophotometrically by the quinolyl hydrazine method described by ROBINS *et al.* (1956). GDH was determined fluorimetrically (LOWRY *et al.*, 1956a). G6DH was measured by a modification of a method developed by Dr. R. WAYNE ALBERS (personal communication). Glucose-6-phosphate was oxidized with triphosphopyridine nucleotide (TPN^+) and the TPNH formed was measured fluorimetrically rather than spectrophotometrically as in the original procedure. Since TPNH gives a satisfactory reading relative to the blank at a concentration of 10^{-7} M, the sensitivity of the method is increased a hundredfold. The substrate reagent

VOL.
1
66/57

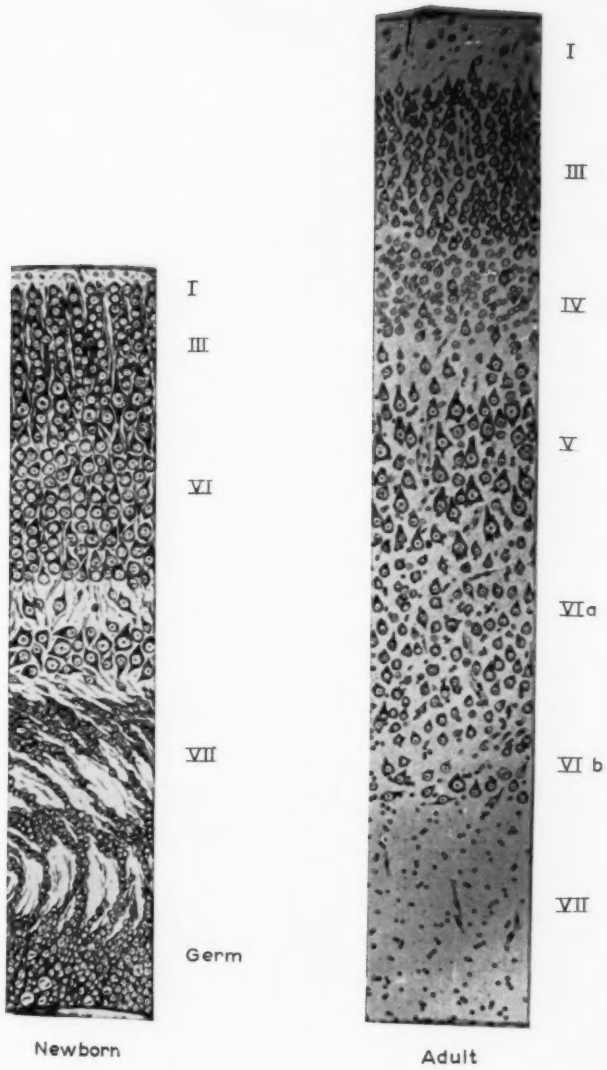


FIG. 1.—Cross-sections of the newborn and adult rat cerebral cortex. (Reproduced from original Figs. 8 and 9, SUGITA, N., *J. Comp. Neurol* (1917) **28,** 538, by permission of the publisher.)

facing p. 174

VOL
1
1956/

contained 0·1 M 2-amino-2-methyl propanol buffer pH 8·93, 5·45 mM glucose-6-phosphate (sodium salt, Sigma Chemical Co. St. Louis), 1 mM ethylenediaminetetra-acetic acid and 10 mM magnesium chloride. This reagent was stored frozen. Just before use 20 mM TPN^+ (Sigma) was added to make a final concentration of 2 mM. The stock TPN^+ solution was also stored frozen.

Tubes (1·6 to 1·8 mm bore, 55 mm long), each containing a tissue sample of 0·3 to 1 μg dry weight, were placed in a rack in an ice bath and 2 μl of substrate were added without mixing. After 5 to 10 min each tube was gently tapped to mix the contents and the rack transferred to a 38° water bath for one hour. After returning the rack to the ice bath the reaction was stopped with 20 μl of 0·02 N sodium hydroxide solution and the tubes were shaken by vibration. Aliquots of 19 μl were removed and placed in 150 μl volumes of 0·01 N sodium hydroxide solution contained in special fluorimeter tubes. These tubes were 4 mm in bore and 70 mm long with flat bottoms. They were carefully selected for uniformity and were used with a special adapter (Lowry *et al.*, 1954a) in the Farrand fluorimeter. The fluorescence was read within 2 hr against a quinine working standard using a primary filter of Corning glass No. 5860 (to isolate the 365 mμ Hg line) and, as secondary filter, Corning glass Nos. 4308, 5562 and 3387.

Blanks were provided as well as DPNH standards consisting of 2 μl of complete reagent which contained added DPNH to provide a concentration 1 mM.

Comments on G6DH *Method.* DPNH (Sigma) was used as a standard. TPNH was prepared from TPN^+ by reduction with glucose-6-phosphate and G6DH. The fluorescence was found to be within 10 per cent of that calculated from DPNH. It has since been found that Mg^{++} at a concentration of 0·2 mM and over enhances the fluorescence of TPNH in alkaline solution (Lowry *et al.*, 1956). The fluorescence of DPNH is much less affected by high Mg^{++} concentrations and theoretically might not be a good standard. However under the analytical conditions described the final Mg^{++} concentration is too low to cause an appreciable error. The blank readings were chiefly due to fluorescence of the TPN^+ preparation. In 0·01 N sodium hydroxide solution the TPN^+ used gave less than one half per cent of the reading of the same amount of TPNH. Brain tissue contributes negligibly to the blank. The reaction velocity with DPN^+ was only 5 per cent of that with TPN^+. Little or no TPN^+ reduction occurred if either glucose or fructose diphosphate was substituted for glucose-6-phosphate. The composition of the buffer is designed to provide a near maximal velocity for G6DH of rat brain, and it is the result of preliminary studies with different levels of substrate and TPN^+, and at different pH's.

RESULTS

In whole brain homogenates prepared from rats of 0 to 7 days there was found to be little change in any enzyme measured except GDH (Fig. 2). Between 7 days and maturity there occurs a 4-fold increase in MDH, a 2·7-fold increase in GDH, a 2-fold rise in LDH and almost no change in G6DH. These changes are reported on the basis of *wet weight*. Since the proportion of dry weight is increasing over this period of growth, the changes are less striking on a dry weight basis. From birth to

TABLE 1.—RATIOS BETWEEN ENZYME ACTIVITIES OF ALL LAYERS AND ACTIVITIES OF WHOLE ADULT BRAIN HOMOGENATES

All activities are calculated on the basis of total dry weight. Each layer of newborn (NB), ten-day-old (10D), and adult cortex is compared with the average value for adult whole brain.

Cell layer	GDH			MDH			LDH			G6DH		
	NB	10D	Adult	NB	10D	Adult	NB	10D	Adult	NB	10D	Adult
I	0·36	1·24	1·24	0·62	0·72	1·05	1·75	1·27	2·01	1·55	1·62	1·38
III	0·19	0·84	0·89	0·71	0·77	1·07	1·45	1·77	1·83	1·61	1·25	1·41
IV			0·86			0·82			1·79			1·32
V		0·81	0·79		0·67	1·08		1·84	1·32		1·44	1·08
VI	0·21	0·72	0·57*	0·58	0·75	0·49*	1·14	2·08	1·52*	1·58	1·42	1·13*
VIb			0·47			0·21			1·10			0·95
VII	0·21	0·68	0·28	0·55	0·47	0·12	1·39	1·59	1·07	1·58	1·13	0·81
Germinal	0·23			0·54			1·73			1·59		
Whole brain	0·35	0·64	(1·00)†	0·38	0·70	(1·00)	0·76	0·93	(1·00)†	1·71	1·33	(1·00)†

* Layer VIa.
† The activities in whole adult brain were respectively 3·88, 62·5, 21·1, and 1·07 moles per kg total dry weight per hour.

maturity there is about a threefold increase in GDH and MDH, a 33 per cent increase in LDH and a marked *fall* in G6DH activity (Table 1).

These changes in the whole brain are reflected to a certain degree in the individual layers, although quantitatively there are major differences (Table 1, Figs. 3, 4, and 5). All cortical layers show a marked increase in GDH (Fig. 3) in the first 10 days of life with little subsequent change, even though the activity in the brain as a whole

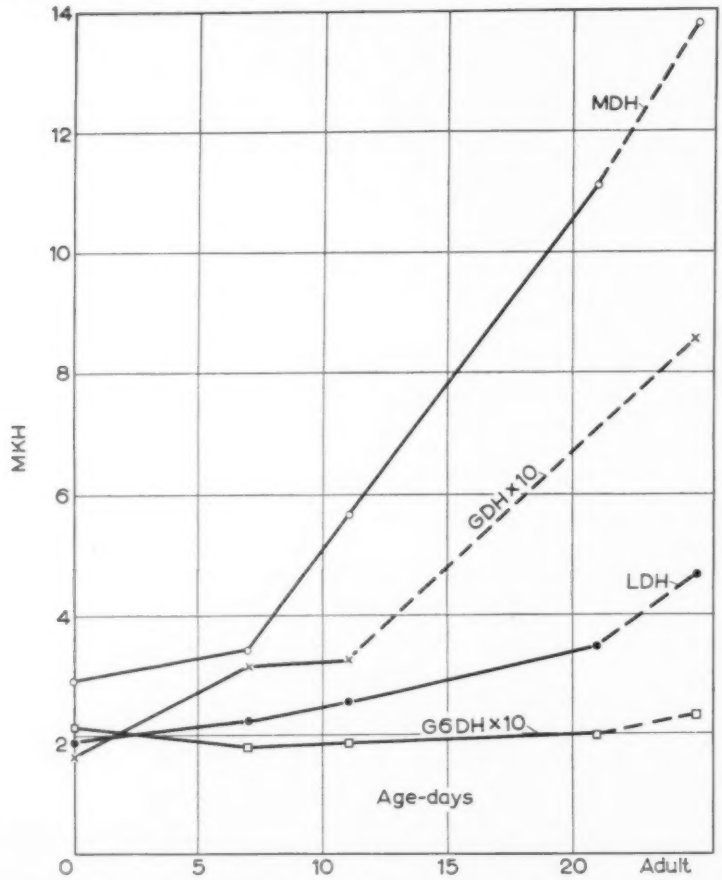


FIG. 2.—Change in enzymatic activity of whole rat brain during postnatal development. Analyses from a total of ten rat brains are represented. Values are expressed in moles of substrate oxidized per kg wet weight per hour.

continues to increase. It is of interest that layer I, which has few nerve cell bodies, is relatively rich in GDH at all three ages and that GDH increases in the other layers when the contribution of cell bodies to the total volume is decreasing (Fig. 3). Clearly GDH is not generally present in high concentration in nerve cell bodies.

MDH scarcely changes in any cortical layer between 0 and 10 days (Fig. 4). Thereafter it increases in the outer layers and decreases in the deeper layers. The concentration of this enzyme in layer VII of the adult rat cortex is especially low.

The concentration of LDH changes with growth in the same direction as MDH,

VOL
1
1956/

OL.
1
6/57

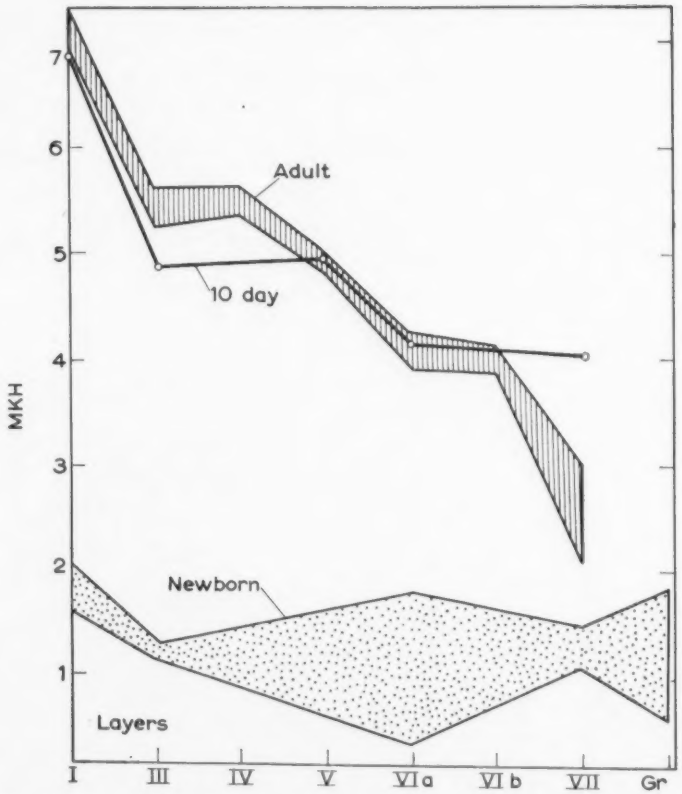


FIG. 3.—Glutamic dehydrogenase activity in the layers of the rat cerebral cortex at three ages. Values are expressed as moles of substrate oxidized per kg lipide-free dry weight per hour. Thickness of the bands indicates ±one standard error. Values for adjacent layers are connected for the sake of visualization, but this does not imply a gradual transition from one layer to the next. Standard error omitted for clarity from the 10-day results, which had approximately the same dispersion as the adult value.

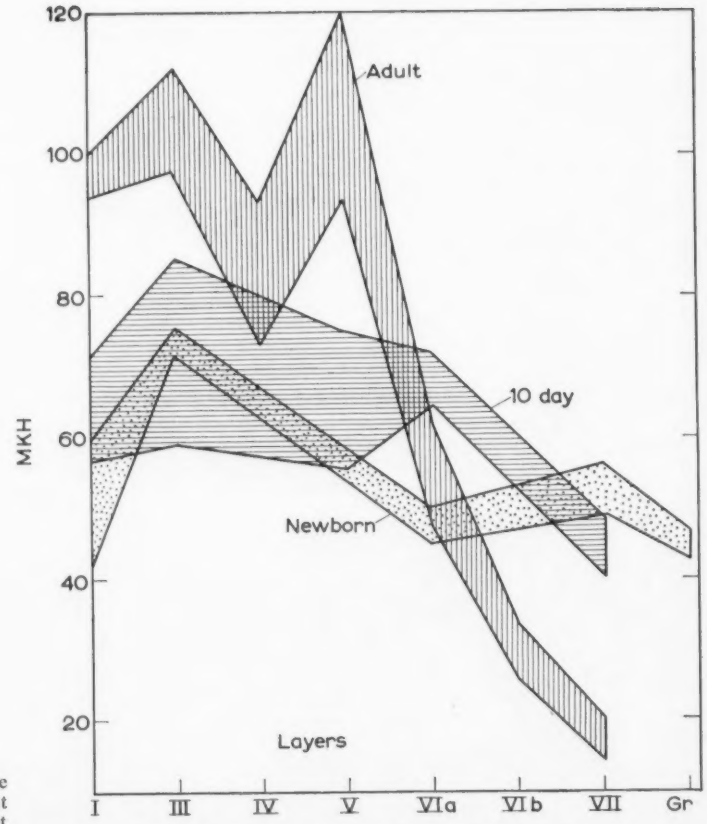


FIG. 4.—Malic dehydrogenase activity in the layers of the rat cerebral cortex at three ages. Values are expressed in the same manner as in Fig. 3.

but to a lesser extent (Fig. 5B). However LDH activity doubles in layer VI between birth and the tenth day of life. After 10 days LDH does not increase in the cortex except in the molecular layer. LDH unlike MDH does not show a marked fall in passing from the outer to the inner cortical layers. On a lipide-free basis (Fig. 5B) myelinated layer VII is almost as rich in this enzyme as the nonmyelinated layers.

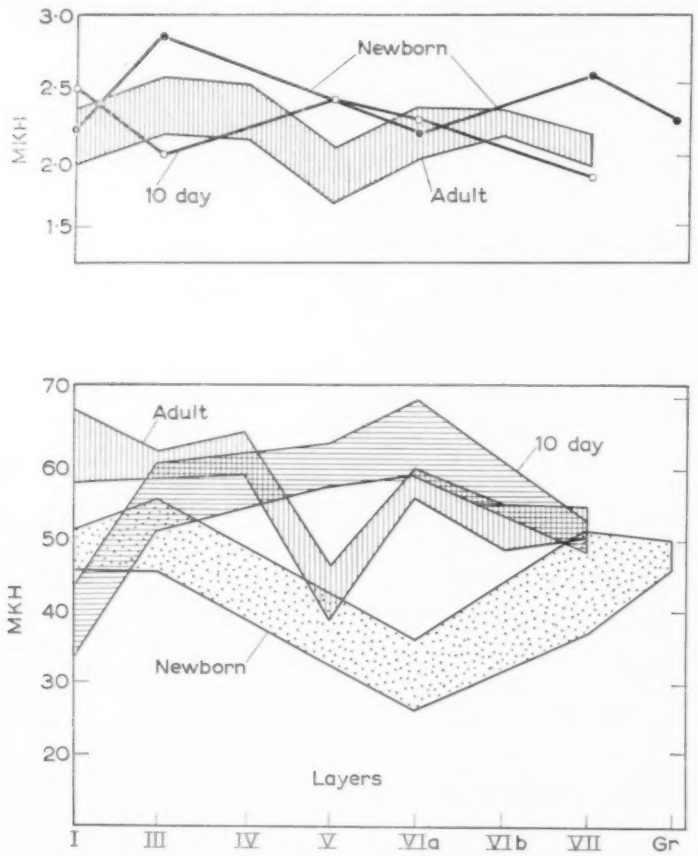


FIG. 5.—(a) Glucose-6-phosphate dehydrogenase (upper portion) and (b) lactic-dehydrogenase activity (lower portion) in the layers of rat cerebral cortex at three ages. Values are expressed in the same manner as in Fig. 3. In the case of glucose-6-phosphate dehydrogenase, the standard errors were about the same for all groups, but for clarity are only shown for the adult.

G6DH shows less change than any of the other three enzymes either with growth or with position in the cortex. This is particularly true on a lipide-free basis (Fig. 5A).

In general, the total brain lipide increases with age (Table 2). The increase is most striking in the deeper layers. Unfortunately the lipide values for the newborn animals are not as reliable as the rest. Owing to fragility of the sections it was difficult to avoid loss of fragments during solvent extraction. Therefore the reported values should be regarded as the maximal lipide content.

VOL
1
1956/

TABLE 2.—DRY WEIGHT PER LITRE, LIPIDE AND LIPIDE-FREE SOLID CONTENT OF THE CORTICAL LAYERS IN THE NEWBORN, TEN-DAY AND ADULT RAT CEREBRAL CORTEX
Each value is the mean of four to ten measurements. The standard error is indicated. The lipide values are calculated on the basis of total solids.

	Lipide* (g per kg dry weight)	Total solids (g per litre)	Lipide-free solids (g per litre)
†Newborn Layer I	206 ± 12 (5)	199 ± 28 (4)	158
Newborn Layer III	390 ± 74 (4)	178 ± 17 (5)	109
Newborn Layer VIa	220 ± 70 (5)	156 ± 7 (4)	122
Newborn Layer VII	344 ± 74 (5)	163 ± 20 (3)	107
Newborn Layer Germ.	228 ± 111 (6)	202 ± 29 (5)	156
†Ten-day Layer I	300 ± 54 (5)	217 ± 46 (4)	152
Ten-day Layer III	357 ± 35 (7)	167 ± 19 (4)	108
Ten-day Layer V	361 ± 22 (4)	197 ± 32 (4)	126
Ten-day Layer VIa	319 ± 4 (5)	178 ± 15 (4)	121
Ten-day Layer VII	360 ± 36 (5)	143 ± 24 (4)	92
Adult Layer I	328 ± 20 (4)	278 ± 18 (5)	189
Adult Layer III	366 ± 19 (4)	277 ± 26 (5)	175
Adult Layer IV	392 ± 13 (4)	273 ± 7 (5)	166
Adult Layer V	371 ± 11 (3)	271 ± 3 (4)	170
Adult Layer VIa	445 ± 21 (6)	277 ± 6 (5)	154
Adult Layer VIb	554 ± 15 (4)	322 ± 5 (4)	144
Adult Layer VII	580 ± 14 (5)	397 ± 6 (5)	167

* Lipide plus other alcohol-soluble solids.
† The standard errors found with samples of the ten-day and newborn age groups are high owing to the fragility and fragmentation of the extracted samples, and the values tend to err on the low side.

In addition to the changes in lipide, there occurs an increase in the concentration of lipide-free solids between 10 days and maturity, i.e., there is a decrease in the water content. Evidently as the cells grow and processes proliferate there is an increase in the dry weight of the tissue.

DISCUSSION

It is believed that the four enzymes measured are indicative of four distinct types of metabolism. Thus MDH, LDH, G6DH, and GDH may indicate respectively the capacity for (a) oxidation through the citric acid cycle, (b) glycolysis, (c) metabolism through the oxidative "shunt" and (d) oxidation or synthesis of glutamate. Further experiments will be necessary to prove this hypothesis; nevertheless, the point of view may be useful as a guide to further experiments. On this assumption, during maturation the nonmyelinated cortical layers increase greatly in ability to utilize glutamate and to a lesser extent in capacity for oxidation through the citric acid cycle. At the same time there is comparatively little change in glycolysis or in direct glucose oxidation.

The rise in GDH occurs exclusively during the first 10 days. This suggests that glutamate metabolism is especially active in nerve-cell processes which proliferate greatly in this 10-day interval. The finer arborizations may be especially involved since the molecular layers both in Ammon's horn (STROMINGER and LOWRY, 1955) and cerebral cortex (ROBINS, SMITH *et al.*, 1956) are especially rich in GDH.

However, as the molecular layer of the cerebellum is only moderately active in GDH (ROBINS, ROBERTS *et al.*, 1956) it may be that only certain kinds of cell processes emphasize the metabolism of glutamate. It is also possible that glia are rich in GDH.

The distribution of MDH, LDH, and GDH within the adult rat cortex is very similar to that found in the monkey (ROBINS *et al.*, 1956).

There is a moderate decrease in the ratio of MDH to LDH with maturation which is in keeping with the numerous reports that glycolysis is relatively prominent in embryonic or immature animals. The change in ratio seems insufficient, however, to explain the great capacity of young animals to withstand anoxia. Thus FAZEKAS (1951) found that the survival time of rats in nitrogen was 50 minutes at birth, 10 minutes at 10 days and only 3 minutes at 18 days or in the adult.

SUMMARY

1. Malic, lactic, glutamic, and glucose-6-phosphate dehydrogenases were measured in brain homogenates of newborn, 7-, 11-, and 21-day-old, and adult rats.

2. These enzymes were measured also in each of the major cortical layers of newborn, ten-day-old and adult rats. Glutamic dehydrogenase increases markedly in all cortical layers between birth and 10 days and changes little thereafter. Between 10 days of age and maturity malic dehydrogenase activity increases in the outer cortical layers and decreases in the inner layers. Lactic dehydrogenase activity undergoes much smaller changes in the same direction, whereas glucose-6-phosphate dehydrogenase activity in all layers remains almost unchanged in relation to the lipide-free dry weight.

3. Between 10 days and maturity total lipide increases and water content decreases in all cortical layers.

REFERENCES

CRAIN S. M. (1952) *Proc. Soc. Exp. Biol., N.Y.* **81,** 49.

DONALDSON H. H. and HATAI S. (1911) *J. Comp. Neurol.* **21,** 417.

FAZEKAS J. F., ALEXANDER F. A., and HIMWICH H. E. (1951) *Amer. J. Physiol.* **134,** 281.

FLEXNER J. B. and FLEXNER L. B. (1948) *J. Cell. Comp. Physiol.* **31,** 311.

FLEXNER L. B. and FLEXNER J. B. (1950) *J. Cell. Comp. Physiol.* **36,** 351.

FLEXNER L. B. (1951–52) *Harvey Lectures* Series **47,** 156.

FLEXNER L. B., BELKNAP E. L., and FLEXNER J. B. (1953) *J. Cell. Comp. Physiol.* **42,** 151.

LOWRY O. H. (1953) *J. Histochem. and Cytochem.* **1,** 420.

LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M. L., and FARR A. L. (1954) *J. Biol. Chem.* **207,** 1.

LOWRY O. H., ROBERTS N. R., WU M. L., HIXON W. S., and CRAWFORD E. J. (1954) *J. Biol. Chem.* **207,** 1.

LOWRY O. H., ROBERTS N. R., and LEWIS C. (1956) *J. Biol. Chem.* **220,** 879.

LOWRY O. H., ROBERTS N. R., KAPPHAHN J. I., and LEWIS C. (1956) *Fed. Proc.* **15,** 304.

PETERS V. B. and FLEXNER L. B. (1950) *Amer. J. Anat.* **86,** 133.

POTTER V. R. (1945) *Cancer Res.* **5,** 21.

ROBINS E., ROBERTS N. R., EYDT K. M., LOWRY O. H., and SMITH D. E. (1956) *J. Biol. Chem.* **218,** 897.

ROBINS E., SMITH D. E., EYDT K. M., and MCCAMAN R. E. (1956) *J. Neurochem.* **1,** 68.

STROMINGER J. and LOWRY O. H. (1955) *J. Biol. Chem.* **213,** 635.

SUGITA N. (1917) *J. Comp. Neurol.* **28,** 495.

SUGITA N. (1918) *J. Comp. Neurol.* **29,** 11.

WAELSCH H. (1955) *Biochemistry of the Developing Nervous System* Academic Press, Inc., New York.

VOL 1 1956/

Journal of Neurochemistry, 1956, Vol. 1, pp. 181–191, Pergamon Press Ltd., London.

FACTOR I—INHIBITORY FACTOR FROM BRAIN

ASSAY. CONDITIONS IN BRAIN. SIMULATING AND ANTAGONIZING SUBSTANCES

K. A. C. ELLIOTT* and ERNST FLOREY*
Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute
and the Department of Neurology and Neurosurgery, McGill University,
Montreal, Canada

VOL.
1
56/57

(*Received* 19 *September* 1956)

INTRODUCTION

EXTRACTS from brain or spinal cord of mammals contain a factor which reversibly prevents the generation of impulses by a stretch receptor neuron of the crayfish, whether the receptor is stimulated by stretch or by the application of acetylcholine (FLOREY, 1953, 1954a). The agent (or agents) responsible for this inhibition has been called "Factor I" and has been partially purified by a variety of methods (FLOREY, 1954a; FLOREY and MCLENNAN, 1955a, 1955b). In crustaceans such purified preparations block neuromuscular transmission, slow or stop the neurogenic heart-beat, inhibit the spontaneous activity of the intestine, and counteract the effects of acetylcholine, adrenaline, and nor-adrenaline on the intestine (FLOREY, 1954a, 1954b). They antagonize or mimic the effects of acetylcholine on various other invertebrate preparations (FLOREY, 1956). Partially purified extracts have been found to inhibit spontaneous and acetylcholine-stimulated contractions of guinea-pig and rabbit intestine (FLOREY, 1953), to block synaptic transmission in the inferior mesenteric ganglion of cat and rabbit and in the stellate ganglion of the cat (FLOREY and MCLENNAN, 1955a, 1955c) and, if topically applied to the spinal cord of the cat, to block the monosynaptic-tendon jerk-reflex and often to enhance the polysynaptic flexor-reflex. No effect was observed on the superior cervical ganglion nor on neuromuscular transmission in the cat; the hypoglossal nucleus was stimulated (FLOREY and MCLENNAN 1955b, 1955c).

The crayfish stretch-receptor preparation was used in previous work for detecting and estimating the activity of tissue extracts and Factor I preparations. The present report describes the use of the stretch receptor for the quantitative assay of Factor I. The use of the assay method is illustrated by showing the yields of Factor I obtained in some reliable steps in purification. Certain substances, of widely varying chemical composition, have been found to exert effects similar to that of Factor I. Other substances of considerable physiological and pharmacological interest prevent the action of Factor I. Factor I is shown to be present in brain in an "occult" inactive form, from which it is readily released, and to be produced by brain suspensions.

* Aided by grants from the Banting Research Foundation and from Merck and Co., Inc., Rahway, New Jersey, U.S.A.

The method of assay here described is being applied in work on the isolation of the factor and in studies on its neuroanatomical distribution and its biochemical production and destruction.

METHOD OF ASSAY

The test preparation

Large crayfish, of the species *Cambarus virilis*, from biological supply houses have been used in this work. Inhibitory effects of Factor I have also been observed on the stretch receptors of other species of crayfish (*Cambarus clarkii*, *Astacus fluviatilis*, *Pacifastacus sp.*).

The arrangement for recording impulses from stretch receptor neurons is modified from that described by WIERSMA, FURSHPAN, and FLOREY (1953) and depicted by FLOREY (1954a). The dorsal half of the abdomen is removed from the crayfish and the anterior segment of its exoskeleton is held by an alligator clip which is fixed to the edge of a Petri dish. Posterior segments rest on an inclined surface such as a wooden tongue depressor resting across the edge of the dish. The degree of bending of the exoskeleton and consequent stretch of the receptor muscles can be adjusted by changing the position of the inclined surface. The exoskeleton forms a basin which is filled with a saline medium (see below) during dissection, and with this or test solutions during the assay.

A stretch receptor organ with about 4 mm of its nerve is dissected free in one segment with the aid of a dissecting microscope. Details of the dissection have been described by WIERSMA *et al.* (1953). Each stretch receptor organ contains two receptors and their sensory neurons, and the axones from both neurons are present in the nerve. One of these neurons, "cell 1" of ALEXANDROWICZ (1951), discharges continuously if moderate stretch is maintained (WIERSMA *et al.*, 1953). Only effects on this neuron are observed in the assay. The other neuron discharges, only briefly, if the receptor is strongly stretched. Major muscle masses and arteries should be stripped from all segments, and irrelevant muscles and the hypodermis should be removed from the segment containing the stretch receptor to be used. This is necessary in order that wash and test solutions may be changed as quantitatively as possible. The preparation remains on the microscope stage for convenience in making adjustments.

The working space is shielded by a grounded metal cage. The clip holding the crayfish shell is grounded to the cage. The nerve is laid over a platinum-wire electrode, the position of which can be adjusted in three dimensions by an arrangement of worm gear and racks. The electrode is connected to an a.c. amplifier, inside the cage. The amplified impulses are fed into an oscilloscope and, via a power amplifier, into a loud speaker.

With practice, the preparation can be set up within 10 min. Provided the sensitive structures are kept moist, the preparation will usually continue active for hours. If a receptor fails, another can be dissected in another segment of the same animal. Solutions are applied to and removed from the basin formed by the exoskeleton by means of a dropping pipette.

Assay procedure

Excess solution is removed and the activity of the neuron is observed. Then about 0·5 ml of the solution to be tested is applied and mostly removed, and the activity is again observed. If the response is doubtful, this solution is applied and removed

VOL
1
1956/

again. Finally, the fluid in the basin is rinsed with the saline medium and the reversibility of any effect of the previous solution is ascertained. Strong crude extracts from unboiled brain, in which Factor I activity is low (see below), often cause excitation, but block at still higher concentrations. More active extracts and purified solutions do not show this excitatory effect. In low concentration they have no obvious effect, in near-blocking concentration they cause some slowing of the discharges or a brief block followed in a few seconds by gradual recovery of activity, and in higher concentration they block for a longer time or until rinsed off. These different effects are obtained with remarkably small differences in the concentration of the factor. The frequency with which the neuron is firing in the absence of inhibitor does not appreciably affect the concentration of Factor I which is required just to block the activity of the neuron. It has been found most satisfactory to adjust the stretch on the receptor muscle, by adjusting the bend of the exoskeleton, so that the rate of firing is about five per second. A "minimum blocking concentration" of Factor I is defined as the lowest concentration which just blocks the activity of the neuron for 10 sec. Such a solution can be definitely differentiated from one which contains only 10% less of the inhibitory factor.

OL.
1
6/57

Unknown solutions are adjusted to about pH 6·5 and then diluted, with the saline medium (see below), by factors of 10 until a concentration is found which does not block. The lowest of the dilutions which still blocks is then diluted by varying amounts until two concentrations within 10% of each other are found, one of which does not block for as long as 10 sec and one which blocks for at least 10 sec on repeated application. Tests with these two dilutions are repeated.

The minimum blocking concentration of a given extract is fairly constant with the majority of receptor preparations. Preparations do, however, vary in sensitivity, and in extreme cases the minimum blocking concentrations of inhibitor with different receptors may differ by a factor of 10. During a series of assays the sensitivity of a preparation sometimes varies, occasionally quite suddenly. (The most frequent change is an increase in sensitivity to the inhibitory factor after many applications.) Samples of a reference extract are therefore assayed before and after each unknown.

Determinations by this procedure are rapid, and eight or ten assays can be completed in 2 hours.

Standard and units

An extract for use as standard is divided into a number of small bottles and stored in the frozen state at −18°C. Such reference solutions have been kept frozen, with repeated thawing and several hours of standing at room temperature, for months without apparent change in activity.

Until the substance or substances responsible for the inhibitory activity can be chemically identified, we define the unit of activity against a particular receptor preparation as a "crayfish unit," or c.u., which is the amount present in 1 ml of a solution containing the minimum blocking concentration. An arbitrary number of "crayfish units reference" or c.u.r., is ascribed to the activity of the reference standard extract. The reference extract* used in most of this work contained about 100 c.u. per ml when assayed against receptor preparations of average sensitivity, and it

* This extract was provided by Dr. ALVA BAZEMORE. Beef brain was boiled and homogenized in water and the suspension dialysed against water. The dialysate was evaporated almost to dryness and the residue was extracted with ethanol. The ethanol extract was evaporated dry and re-dissolved in water.

was therefore taken to contain 100 c.u.r. per ml. The activity of an unknown sample is compared with that of the reference extract on the same receptor preparation and expressed in c.u.r. per ml. Degrees of purification are indicated by the specific activity, which is expressed as c.u.r. per mg of dry weight.

Saline medium

The solution used for bathing the crayfish preparation and diluting extracts and standard solutions is a modification the solution of VAN HARREVELD (1936). To 1 litre of solution containing 12 g NaCl, 0·4 g KCl, 0·5 g $MgCl_26H_2O$, and 1·5 g $CaCl_2$, 100 ml of 0·11 M tris-maleate* buffer, adjusted to give a final pH of 6·5, and 2 ml of 0·4% bromcresol purple, are added. It is convenient to keep a ten-times concentrated solution of the inorganic salts and to dilute this when required. For the assay of tissues it is convenient also to keep a concentrated salts solution containing no K.

The use of the buffer and indicator greatly facilitates check and control of the pH. Phosphate buffer cannot be used because of the high calcium content of the medium. Neither the tris-maleate buffer nor the particular indicator used (see, however, below) affected the responses of the stretch receptor neuron.

In a previous publication (FLOREY, 1954a) it was reported that the inhibitory factor is active only at a pH below 7 and that, above pH 7, brain extracts caused marked excitation. In the present study these observations could not be repeated. With several extracts, prepared in different ways, the minimum blocking concentrations were the same whether the final dilution was made to pH 5·5, 6·5, or 8·0. The reason for the difference from the previous observations is not known, but may be connected with the difference in species of crayfish used. To avoid possible variable effects, all extracts are adjusted to and tested at or near pH 6·5.

Since electrolyte content of crude-tissue extracts, which cannot be exactly controlled, might affect the assays, the effects of some variations in the medium were tested. Medium containing twice the usual concentration of K caused slight excitation of the stretch receptor neuron but did not appreciably affect the assay of Factor I. Four times the concentration of K caused strong, irregular excitation. Lack of K blocked the activity of the neuron. Doubling the concentration of Ca caused brief excitation followed by block, and halving the concentration caused moderate excitation.

Increase of the Ca concentration by 20% increased the sensitivity to Factor I by about 30%, decrease of the Ca by 20% decreased the sensitivity by about 10%. Hypertonicity due to 200 mM excess sodium chloride or acetate caused excitatory effects and unpredictable variations in sensitivity to Factor I. Less hypertonicity, 20 mM, had no obvious effect.

Preparation of extracts for assay

Extracts that contain little salt and concentrations of Factor I too low to permit further dilution, are adjusted to approximately the usual electrolyte concentration by the addition of one-tenth volume of the concentrated salts solution. Extracts containing alcohol or other organic solvents are evaporated dry under reduced pressure, re-dissolved in the usual medium, and adjusted as to pH before further dilution.

For the assay of the total content of Factor I in tissue, the weighed tissue immersed in K-free salts solution and heated in a boiling-water bath for 12–15 min.

* Tris-(hydroxymethyl) amino methane (Sigma 779, Sigma Chemical Co.).

VOL
1
1956/

It is then homogenized and more K-free solution is added to give a suspension containing 100 mg tissue per ml. The electrolyte content of such a suspension does not differ materially from that of the usual medium. This suspension is diluted as necessary with the complete buffered medium and applied directly to the stretch receptor preparation.

The small amounts of acetylcholine in crude extracts of brain do not interfere with the assay. The presence in the usual medium of as much as 75 mμgm per ml, which is more than could be found in a crude brain extract diluted for assay, had no effect on the stretch receptor neuron nor did it affect the assay of Factor I with the species of the crayfish used.

PARTIAL PURIFICATION OF THE FACTOR I*

The following is an example of the results obtained in a series of simple and reliable steps in the concentration and preliminary purification of the inhibitory factor.

Beef brain was obtained fresh from a slaughter-house and kept at −18°C until it was convenient to work it up. (Fresh, unfrozen brain can also be used.) The tissue, 600 g, was cut into pieces 2–3 cm in diameter and dropped into an equal weight of boiling distilled water. The mixture was boiled for about 10 min and then homogenized in a blender. A total activity of 46,500 c.u.r. was found and a dry weight of 144 g (specific activity 0·32 c.u.r./mg).

The suspension, 1050 g, was poured into cellophane dialyser tubing, 3 cm in diameter, and dialysed in a large erlenmeyer flask against four times its volume of distilled water at 4°C for 50 hr. Assays showed that the dialysate contained a total of 36,000 c.u.r. The suspension remaining in the tube contained about 18,000 c.u.r., and was discarded.

The dialysate was concentrated, by boiling under reduced pressure, to 240 ml. The concentrate contained a total of 33,000 c.u.r. and 8·0 g of dry material (specific activity 4·1 c.u.r./mg).

To the concentrated dialysate a 20% solution of lead subacetate was added until no more precipitation occurred; 75 ml were required. The copius precipitate was removed by centrifugation, excess lead was precipitated with H_2S, and remaining H_2S was removed by aeration with nitrogen. The solution obtained was found to contain 24,000 c.u.r. and 6·0 g of dry weight (specific activity 4 c.u.r./mg). Analyses (flame photometer for K and Na) showed the presence of 26 meq of K, 16·6 meq of Na, and 5·2 meq of chloride. These figures indicate that 3·8 g of the total dry weight could be accounted for as potassium and sodium acetate and chloride.

Most of the potassium was removed by precipitation as the perchlorate. Perchloric acid, 3·2 ml of 60% solution, was added. The mixture was kept cold overnight, filtered, evaporated under reduced pressure to about 24 ml, and chilled. The crystals which formed were removed by centrifugation at 0°C. The solution contained 18,500 c.u.r. and 2·9 g of dry weight (specific activity 6·3 c.u.r./mg), 1·1 meq of K, 13·1 meq of Na, and 3·3 meq of chloride.

When the syrup obtained on evaporation of a portion of the above solution was extracted with warm absolute ethanol, only about half of the activity could be brought into solution. The entire activity was readily obtained in solution in methanol.

* Procedures for the purification of Factor I have been described (FLOREY, 1954a; FLOREY and MCLENNAN, 1955a, 1955b). One of these (FLOREY and MCLENNAN, 1955a) had not been consistently successful.

Only slight increases in specific activity (7·2 c.u.r./mg) were achieved with these solvents. The factor was found not to be at all extractable from aqueous solution with ethyl acetate. It is not soluble in dry acetone. Other methods are now being applied to separate the factor from the salts and other substances of similar solubility properties which accompany it in the above methods of purification.

Most of the chemical properties of Factor I at present known are apparent from the above results. It may be added that a preparation has been heated to 100° for 30 min in the presence of 5 N hydrochloric acid and in 5 N sodium hydroxide without any change in the Factor I activity found on subsequent neutralization and dilution.

THE STATE OF FACTOR I IN BRAIN

When cerebral hemispheres from decapitated rats were homogenized in K-free saline medium, without heating, assays indicated low, variable Factor I activity in the suspension (<10 to 40 c.u.r./g tissue). These suspensions had to be tested at such high concentrations that excitatory effects sometimes rendered the assays uncertain. If the tissue was dropped into the K-free medium and heated in a boiling-water bath before homogenization, assay of the suspension obtained indicated the presence of 83 to 130 c.u.r./g of fresh tissue.

When the tissue was homogenized in water, without heating, and the salt concentration was subsequently adjusted by adding concentrated K-free saline medium, immediate assay showed the presence of the same activity as in tissue which had been heated. No increase in activity was produced by heating such a suspension. Direct comparisons of hemispheres also showed that heating and homogenization in saline medium produced the same activity as heating and homogenization in water. If the tissue was homogenized in saline medium without heating and the suspension was then brought to pH 2·4 or to pH 11·4, by the addition of 0·2 ml N HCl or 0·2 ml N NaOH to 5 ml of suspension, and allowed to stand for 30 min before re-neutralizing, much higher activity was found than in the untreated suspension. These observations, which are summarised in Table 1, indicate that Factor I is present in the tissue mostly in an

Table 1.—Effects of conditions on the factor-I activity of suspensions of rat cerebral hemispheres

Treatment of the tissue	Factor I activity found (c.u.r./g tissue)
Heated, then homogenized in saline medium	85 to 130. Avg. 108 (10 determs.)
Heated, then homogenized in water	95, 110, 120
Homogenized in saline medium. (Not heated)*	5 to 40 Avg. 20 (8 determs.)
Homogenized in saline medium. Made to pH 2·4. Stood 30 min	108, 120
Homogenized in saline medium. Made to pH 11·4. Stood 30 min	145, 125
Homogenized in saline medium. Stood 60 min, then heated	140 to 240. Avg. 173 (6 determs.)
Homogenized in water. Stood 60 min. (Not heated)	170, 250
Homogenized in water. Stood 60 min, then heated	160, 270

Saline medium here means K-free medium for the initial homogenization. Further dilutions were made with the complete medium. The salt concentration in the water suspensions was adjusted before assay by adding concentrated salts solution. The acid and alkaline suspensions were neutralized before assay.

* The unheated saline suspension were assayed at various times, up to more than 60 min after preparation.

VOL 1 1956/

"occult" form from which it can be liberated by heat, hypotonic medium, or dilute acid or alkali.

If solid brain was allowed to stand at room temperature before it was heated and homogenized, slightly more Factor I activity was found than is usual with fresh brain. Brain which had been kept for a long period at −18°C also tended to increase in activity, up to 150 c.u.r./g. If the tissue had been allowed to thaw for some hours during such storage, still higher activity was found, up to 200 c.u.r./g.

When a suspension of brain, unheated, was allowed to stand at room temperature, a considerable increase in the Factor I activity occurred. If the suspension had been prepared in saline medium, the increased activity did not become apparent until the suspension had been heated. If the suspension had been prepared in water, the increase in activity could be observed without heating and no further increase was found on heating. These results, also illustrated in Table 1, indicate that the tissue can form extra Factor I, and this is most probably produced initially in the occult form. The increased amounts of the factor were immediately released in hypotonic medium, but another agent, such as heat, was evidently required to release the newly-formed factor if the suspension was isotonic.

It is probable, however, that the situation is more complicated. It has been reported (FLOREY, 1951) that brain tissue can destroy Factor I. Presumably the changes in activity in these suspensions represent the resultant of synthetic and destructive processes. These processes will be studied in more detail.

EFFECTS OF KNOWN SUBSTANCES ON THE STRETCH RECEPTOR RESPONSES

All substances tested were dissolved in the saline medium mentioned above and neutralized, when necessary to pH 6·5, except where otherwise noted.

Substances which, like Factor I, block the stretch receptor neuron

Solutions of two "amino acid mixtures"—acid hydrolysed casein, purified, from Merck and Co., Rahway, New Jersey—were found to block the discharges of the stretch receptor neuron in concentrations of 0·1 and 0·04 mg per ml. No inhibitory activity was exerted by a number of amino-acids (listed below) at a concentration of 1 mg per ml.

In testing pH indicators for use in the buffered saline medium which is used for bathing the stretch receptor preparation, it was found that bromcresol purple and phenol red were quite inert. Bromthymol blue, however, completely blocked the activity of the stretch receptor neuron at concentrations of 0·01 mg per ml or higher. This effect developed more slowly than that of Factor I. The rate of discharge by the neuron gradually fell to zero in about 30 sec. The block was readily reversed by rinsing the receptor. The structures of the three indicators are very similar. Bromthymol blue differs from both the other indicators in possessing isopropyl groups and the thymol group. Leucine and valine, which contain isopropyl groups, were inactive, but thymol, in concentrations greater than 0·02 mg per ml, blocked the neuron gradually and reversibly. Camphor, which is related to thymol, also exerts a blocking effect, as noted below.

Sodium pentobarbital (nembutal), sodium phenobarbital, and sodium thiopental (pentothal) blocked the activity of the stretch receptor neuron at a concentration of 0·1 mg per ml. The effects were readily reversible. Other barbiturates have not yet been tested.

Substances which oppose the action of Factor I

Picrotoxin, at 0·01 mg per ml or higher concentrations, had no obvious effect on the activity of the neuron when applied alone, but it completely prevented the blocking action of Factor I even when this was applied at five or more times the minimum blocking concentration. The same result has been obtained with solutions of the factor prepared in several different ways. The effect was reversible, though, after application of higher concentrations of picrotoxin, prolonged rinsing was necessary to remove the effect completely. These tests were made with solutions at pH 6·5, which is the pH routinely used for Factor I assay. When rinse and test solutions were made to pH 7·7–8·0, the blocking action of Factor I, applied alone, was unaffected (at least with the species of crayfish used), but picrotoxin then did not prevent this blocking action.

The blocking action of the barbiturates, bromthymol blue and thymol, were not affected by the presence of picrotoxin.

Pentamethylenetetrazole (metrazol) had no effect at low concentrations, but, at 10 mg per ml, although ordinary activity of the neuron was not affected, the effect of Factor I was prevented even at five times the minimum blocking concentration. This action was less effective at pH 8 than at pH 6·5.

Strychnine nitrate, 0·5 mg per ml, had no obvious effect on the activity of the stretch receptor nor on its inhibition by Factor I. (At pH 7·7, repeated applications of strychnine solutions seemed to cause reversible impairment of conduction in the nerve.)

Camphor, one-fifth saturated solution (about 0·2 mg per ml), blocked the activity of the neuron. Lower concentrations had mixed excitatory and blocking effects. The blocking action of Factor I was not prevented by concentrations of camphor which did not themselves block.

Ethanol, 1 mg per ml, alone had perhaps a slight excitatory effect, but it prevented the blocking action of at least twice the minimal blocking concentration of Factor I. This effect was readily reversible.

Ethyl ether behaved in the same way as ethanol. Sodium salicylate also behaved similarly. At 0·1 mg per ml it had no obvious effect alone, but still prevented the action of twice the minimal blocking concentration of Factor I.

Substances which stimulate the stretch receptor neuron

In previous work it was shown that *acetylcholine* can stimulate the activity of the stretch receptor neuron (WIERSMA *et al.*, 1953) and that its effect, like the spontaneous activity, can be blocked by Factor I (FLOREY, 1954a). With the species of crayfish used in the present work, the effect of acetylcholine was apparent with concentrations of 0·001 mg per ml or greater. Acetyl-β-methylcholine was less active. Eserine sulphate and atropine sulphate, 1 mg per ml, also caused some excitation.

5-Hydroxytryptamine creatine sulphate,* at 1 mg per ml had no obvious effect

* Kindly provided by Dr. I. H. PAGE.

VOL
1
1956/

on the stretch receptor of the species of crayfish used in the present study nor on the sensitivity to Factor I. In previous work[5] with a different genus of crayfish, *Astacus fluviatilis*, 5-hydroxytryptamine creatine sulphate was found to cause excitation of the stretch receptor neuron and of sensory cells in the joints of the chelipeds.

As mentioned earlier, excitatory effects can be produced by strong, unheated brain suspensions, by ion and osmotic imbalance, and by camphor, ethanol, ether, and salicylate. Slight excitations have been noted with a number of other substances, but these were difficult to differentiate from surface tension and mechanical effects on the stretch receptor muscle.

In a previous study (FLOREY, 1954b) in which a different species of crayfish, *Cambarus clarkii*, was used, it was found that heated brain and spinal cord extracts and purified preparations, when applied at a pH above 7, caused marked excitation of stretch receptor neurons. In the presence of picrotoxin this excitation became apparent also at a pH below 7. Strychnine potentiated the excitation above pH 7. These marked excitatory effects have not been observed in the present work.

OL.
1
6/57

Substances which exert no obvious effect

The following substances have been tested at a concentration of 1 mg per ml and were found to have no obvious effect on the stretch receptor preparation: DL-alanine, DL-α-aminobutyric acid, L-arginine, L-aspartic acid, L-asparagine, DL-citrulline, L-cysteine, L-cystine, glycine, L-glutamate, L-glutamine, L-histidine, DL-homocysteine, L-hydroxyproline, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-norleucine, DL-norvaline, L-ornithine, L-proline, DL-phenylalanine, L-sarcosine, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, 3,5-di-iodo-L-tyrosine, L-valine, acetyl tryptophane, taurine, *p*-aminobenzoic acid, creatine, creatinine, betaine, nicotinic acid, D-calcium pantothenate, thiamine pyrophosphate, adenine, thymine, xanthine, 2-amino-4-methyl pyrimidine.

The following substances, 1 mg per ml, were tested on the activity of the stretch receptor, and on the sensitivity of the receptor to Factor I, and were found to cause no obvious changes: L-adrenaline bitartrate, L-noradrenaline bitartrate, thiamine chloride, histamine, choline chloride, sodium acetate, sodium adenosine-5-phosphate, sodium adenosine diphosphate, sodium adenosine triphosphate, calcium creatine phosphate, heparin, sodium L-glutamate, L-glutamine, L-asparagine, glutathione, 5-hydroxytryptamine creatine sulphate (see, however, above).

DISCUSSION

The nature of the "occult" form of Factor I is not obvious; it could be a precursor which is readily changed to Factor I or a loose inactive complex, but the factor may be occluded, in the intact tissue, in some subcellular particle or vesicle. The latter possibility seems the most likely to us. It seems probable that nearly all of the Factor I is present in the tissue in the occult form and that the small variable activity found in the unboiled saline suspensions had been released by mechanical action during homogenization. The situation is very similar to that which applies to acetycholine, which is known to be present in the tissue in a precursor, bound or occluded form from which it can be released by organic solvents, weak acid (MAUN *et al.*, 1938), weak alkali (with destruction), hypotonic medium, or mechanical action (BRODKIN and ELLIOT, 1953). The adrenaline and nor-adrenaline of bovine adrenal medulla

have also been shown to be associated with granules in a condition in which they are not able immediately to exert their full biological action (BLASCHKO *et al.*, 1955).

The crayfish stretch receptor seems to be a fairly specific indicator of the inhibitory action of Factor I. Several known pharmacological agents can inhibit the activity of the stretch receptor neuron, but none are as active as preparations which have recently been obtained from brain (BAZEMORE, FLOREY, and ELLIOTT, to be published) and it seems significant that none behaves like Factor I in rapidity of action and the prevention of its action by picrotoxin. The considerable inhibitory activity of acid casein hydrolysate is somewhat surprising, particularly since none of the L-amino acids tested were found to be active and Factor I activity has not been found in tissues other than brain and spinal cord (FLOREY and MCLENNAN, 1955a).

One of the most interesting results of our experiments is the finding that picrotoxin specifically inhibits the inhibitory action of Factor I. BRADLEY, EASTON, and ECCLES (1953) reported that strychnine blocks the action of inhibitory neurons in the spinal cord of the cat, and they discussed the possibility that removal of inhibitory influences might underlie the various effects of strychnine. Previous observations and the present results are in accord with the hypothesis that Factor I may be a transmitter substance of inhibitory neurons and that interference with its action may be the basis of the convulsive effects of some drugs.

The intravenous injection of strychnine has been found to prevent the inhibitory action on the extensor reflex which is obtained when preparations containing Factor I are topically applied to the spinal cord of the cat, and subcutaneous injections of such preparations* have been found to protect mice from the convulsive or lethal effect of strychnine (FLOREY and MCLENNAN, 1955).

The crayfish is very sensitive to the convulsant action of picrotoxin (FLOREY, 1951), and this substance, in low concentration, prevents the inhibitory action of Factor I on the stretch receptor. Metrazol, in large doses, will induce convulsions, and relatively high concentrations of metrazol prevent the action of Factor I on the stretch receptor. In fact, the potencies of these drugs as convulsants in the crayfish (FLOREY, 1951) are roughly inversely proportional to the concentrations of picrotoxin and metrazol required to prevent the action of Factor I. The crayfish is not sensitive to the convulsant action of strychnine and this substance does not obviously affect the stretch receptor not its inhibition by Factor I. Camphor, 0·5 ml of a saturated solution injected into the crayfish heart, produced no convulsion. Camphor does not prevent the action of Factor I on the stretch receptor.

The observations that ethanol, ethyl ether, and salicylate prevent inhibition by Factor I and that barbiturates themselves inhibit, offer bases for pharmacological speculation. It is commonly stated that alcohol "removes inhibitions"! The initial excitatory effects of ether when administered for general anaesthesia might result from suppression of transmission of inhibitory impulses. That salicylate should also prevent the action of Factor I seemed surprising, but it is known that excitatory states and convulsions can be produced by salicylates in humans. The inhibition of the receptor by the barbiturates accords well with their depressant properties.

* Preliminary results now suggest that there is more than one substance obtainable from brain which affects the crayfish stretch receptor. Not all preparations have been found, by the method used, to protect mice against strychnine convulsions.

VOL 1 1956/

SUMMARY

(1) A rapid and accurate method for assaying the activity of Factor I in tissue and in purified tissue extracts by means of the stretch receptor organ of the crayfish is described.

(2) Steps in the purification of Factor I from beef brain are described, with estimates of yields.

(3) Factor I is present in brain in a form in which it is not active in inhibiting the spontaneous discharges of the crayfish stretch receptor neuron. It can be released in the active form by heat, weak acid or alkali, or hypotonic solution.

(4) Brain suspensions increase their content of Factor I with time.

(5) The spontaneous discharges of the crayfish stretch receptor neuron can be blocked by purified casein hydrolysates, by bromthymol blue, thymol, and camphor and by pentobarbital, phenobarbital, and thiopental. These substances are not as active as purified preparations of Factor I which have been obtained from brain.

(6) Picrotoxin, at low concentrations, does not obviously affect the spontaneous activity of the stretch receptor neuron, but prevents the inhibitory effect of Factor I. It does not prevent the effect of the thymol derivatives or barbiturates. Metrazol has a similar effect to that of picrotoxin, but only in much higher concentrations. Strychnine has no effect. Camphor at less than its inhibitory concentration has excitatory effects and does not prevent the action of Factor I. Picrotoxin and, at high dosage, metrazol are convulsive agents in the crayfish; strychnine and camphor are not.

(7) Ethanol, ethyl ether, and salicylate, at concentrations which have little effect on spontaneous activity, also prevent the action of Factor I on the stretch receptor.

(8) A variety of other substances of biochemical and physiological interest were found to have no obvious effect on the stretch receptor.

We are grateful to Merck and Co. Inc., USA, for supplying us with samples of many of the compounds tested.

REFERENCES

FLOREY E. (1953) Ueber einen nervoesen Hemmungsfaktor in Gehirn und Rueckenmark. *Naturwissenschaften* **40,** 413–414

FLOREY E. (1954a) An inhibitory and an excitatory factor of mammalian brain and their action on a single sensory neuron. *Arch. int. Physiol.* **62,** 33–53.

FLOREY E. and MCLENNAN H. (1955a) The release of an inhibitory substance from mammalian brain and its action on peripheral synaptic transmission. *J. Physiol.* **129,** 384–392.

FLOREY E. and MCLENNAN H. (1955) Effects of an inhibitory factor (Factor I) on central synaptic transmission. *J. Physiol.* **130,** 446–455.

FLOREY E. (1956) The action of factor I on certain invertebrate organs. *Canad. J. Biochem. Physiol.* **34,** 669–681.

FLOREY E. (1954b) Ueber die Wirkung von Acetylcholin, Adrenalin, Faktor I und anderen Substanzen auf den isolierten Enddarm des Flusskrebses *Cambarus clarkii Girard. Z. vergl. Physiol.* **36,**1–8.

FLOREY E. and MCLENNAN H. (1955) Die Wirkung des Hemmungsfaktors aus Gehirn and Rueckenmark auf periphere und zentrale synaptische Uebertragung. *Naturwissenschaften* **42,** 51–52.

WIERSMA C. A. G., FURSCHPAN E., and FLOREY E. (1953) Physiological and Pharmacological Observations on Muscle Receptor Organs of the Crayfish, *Cambarus clarkii* Girard. *J. exp. Biol.* **30,** 136–150.

ALEXANDROWICZ J. S. (1951) Muscle Receptor organs in the abdomen of *Homarus vulgaris and Palinurus vulgaris. Quart. J. Micr. Sci.* **92,** 163–199.

HARREVELD A. VAN (1936) A Physiological Salt Solution for Freshwater Crustaceans. *Proc. Soc. Exp. Biol. and Med.* **34,** 428–432.

VOL. 1 56/57

FLOREY E. (1951) Vorkommen und Funktion sensibler Erregungssubstanzen und sie abbauender Fermente im Tierreich. *Z. vergl. Physiol.* **33,** 327–377.

MANN P. J. G., TENNENBAUM M., and QUASTEL J. H. (1938) On the Mechanism of Acetylcholine Formation in Brain *in vitro*. *Biochem J.* **32,** 243–261.

BRODKIN E. and ELLIOTT K. A. C. (1953) Binding of Acetylcholine. *Amer. J. Physiol.* **173,** 437–442.

BLASCHKO H., HAGEN P., and WELCH A. D. (1955) Observation on the Intracellular Granules of the Adrenal Medulla. *J. Physiol.* **129,** 27–49.

BRADLEY K., EASTON B. M., and ECCLES J. C. (1953) An investigation of primary or direct inhibition. *J. Physiol.* **122,** 474–488.

VOL
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 193–202. Pergamon Press Ltd., London

THE HISTOCHEMISTRY OF THE ESTERASES OF RAT BRAIN, WITH SPECIAL REFERENCE TO THOSE OF THE HYPOTHALAMIC NUCLEI

W. J. PEPLER* and A. G. E. PEARSE
Department of Pathology, Postgraduate Medical School of London

(*Received* 11 *October* 1956)

VOL. 1 56/57

A CONSIDERABLE number of histochemical investigations has been carried out on the esterases of the nervous system and other tissues of the rat (KOELLE, 1954; KOELLE, 1955; GOMORI and CHESSICK, 1953; RAVIN, ZACKS, and SELIGMAN, 1953; MALATY and BOURNE, 1955). These studies have been concerned with either the cholinesterases (ChE) or the nonspecific esterases. In the first instance, acetylthiocholine (AThCh) and butyrylthiocholine (BuThCh) have been used as substrates, with di-*iso*propyl fluorophosphate (DFP) as the selective inhibitor of pseudo-cholinesterase, according to the method described by KOELLE and FRIEDENWALD (1949) or one of its later modifications (KOELLE, 1950; KOELLE, 1951). In the case of the non-specific esterases the α- or β-naphthyl acetate and naphthol AS acetate methods have been employed. BARRNETT (1952) and BARRNETT and SELIGMAN (1953) have also used indoxyl acetate and butyrate as substrates.

The present histochemical study deals with the hydrolysis of O-acetyl-5-bromo-indoxyl (5-BrI) and a number of the above-mentioned substrates by cold formol-calcium-fixed frozen sections of normal rat brain. An attempt was made to characterize these rat-brain enzymes further by using certain inhibitors and activators before and during the period of hydrolysis and by varying the pH of the incubation mixture. In addition to these studies, variations in the specific or acetylcholinesterase and indoxyl esterase content of the main hypothalamic nuclei were investigated under certain experimental conditions.

MATERIAL AND METHODS

Stock albino and hooded rats were used for all studies.

Tissues

After killing the rats with coal-gas, the brain was removed as a whole and fixed in cold (4°C) neutral 15% formol calcium (1% calcium chloride in 6% aqueous formaldehyde. The pH of this solution, kept over marble chips, is approx. 7·4) for 16–24 hr. Washing was not carried out before cutting, as it was found that this procedure caused marked loss of enzyme, especially the cholinesterases. After fixation, sections were cut on a freezing microtome set at 20 μ; these sections were washed for a few minutes in distilled water before commencing enzymic studies, and free floating sections were used throughout. In all animals, coronal semiserial sections of the brain were cut, extending from the optic chiasma anteriorly to the mamillary bodies posteriorly.

* Nuffield Foundation Fellow.

Substrates

Unless otherwise specified, the methods used were as described by PEARSE (1953). The substrates used were:

(a) O-acetyl-5-bromoindoxyl (5-BrI)
(b) α-naphthyl acetate,
(c) 3-acetoxy-2-naphthoic acid anilide (Naphthol AS),
(d) acetylthiocholine and butyrylthiocholine (KOELLE, 1951; as modified by GOMORI, 1952).

The α-naphthol and naphthol AS methods were, however, soon entirely substituted by the 5-BrI method in this study because of the greater sensitivity of the latter to all the esterases demonstrable by the first two methods. Myristoylcholine was used initially, but discarded because of the inability of rat esterases to hydrolyse this substrate (cf. GOMORI, 1948).

Incubation

With 5-BrI the sections were incubated for 1½ hr and in the case of AThCh the optimum time was found to be 6 hr, at 37°C.

Inhibitors

Sodium fluoride: 10^{-3} M
Sodium taurocholate: 10^{-3} M
Eserine sulphate: 10^{-4} M
62.C.47 (1:5-bis-(4-trimethylammonium-phenyl)pentan-3-one di-iodide): 10^{-4} M
iso-OMPA (tetra-*iso*propylpyrophosphoramide): 10^{-4} M
Mipafox (NN′-di-*iso*propylphosphorodiamidic fluoride): 10^{-3} M
E600 (diethyl-*p*-nitrophenylphosphate): 10^{-5} M
Sodium dodecyl sulphate (SDS): 10^{-3} M
Hydrocinnamic acid: 10^{-3} M
Hydroxylamine: 10^{-3} M
Iodoacetic acid: 10^{-3} M
p-Chloro-mercuric phenyl sulphonic acid: 10^{-4} M
Silver nitrate: 2×10^{-3} M

Ethopropazine hydrochloride (Lysivane), a reversible pseudo-cholinesterase inhibitor, was also tried, but it produced a slight precipitate when mixed with the substrate and was therefore omitted from further investigations.

The sections were first left in the inhibitor + buffer solution for 1 hr and then transferred to the substrate mixture. Reversible inhibitors were included in this mixture, but irreversible inhibitors were left out. Silver nitrate was used as an irreversible inhibitor, in distilled water instead of buffer.

Buffers

The buffer used in the inhibitor studies was always tris(hydroxymethyl)aminomethane. In the pH series, however, we used MICHAELIS' barbital buffer, and sections were incubated for 3 hr at 37°C.

Control slides

In the inhibitor studies, control sections were always incubated in buffer for the same time as the experimental sections in buffer + inhibitor before transferring them to the substrate mixture.

Grading of enzyme activity

The results were graded visually, using the following system:

N = Normal amount of enzyme as compared with control sections.
−1 = 30% reduction
−2 = 60% reduction
−3 = 90% reduction
−4 = total inhibition
+1 = 30% increase
+2 = 60% increase

VOL 1 1956/

RESULTS

These are set out below in tabular form.

TABLE 1.—EFFECT OF INHIBITORS AND ACTIVATORS ON BRAIN ESTERASES

Inhibitor	Pericyte	Choroid Pl.	Neurone*	Pseudo ChE†	Specific ChE†
NaF 10^{-4} M	N	N	N	N	N
Eserine 10^{-4} M	N	N	N	−4	−4
62.C.47 10^{-4} M	N	N	N	N	−4
Iodoacetic Ac. 10^{-3} M	N	N	N	N	N
p-Chloro. MPSA 10^{-4} M	N	N	N	−2	N
Mipafox 10^{-4} M	N	−2	N	−2	−4
E600 10^{-5} M	N	−1	−4	−4	−4
SDS 10^{-3} M	N	N	N	−4	−4
Hydrocinnamic Ac. 10^{-3} M	+1	+1	+1	+1	+1
Hydroxylamine 10^{-3} M	N	N	N	−3	−3
Na-Taurocholate 10^{-3} M	N	N	N	N	N
$AgNO_3$ 2×10^{-3} M	−2	−3	−4	−4	N
iso-OMPA 10^{-4} M	N	N	N	−4	N
Cysteine 10^{-3} M	+2	+	+2	+2	+2

* By "neurone" here is meant the neurones in the cerebral grey matter. The neurones of the hypothalamic nuclei etc. behave like pericytes (see Discussion).

† As indication of inhibition of non-specific cholinesterase (pseudocholinesterase), known sites such as the capillary walls and myelinated areas were taken. The caudate nucleus was used to assess acetylcholinesterase inhibition.

I. *Identification and Localization of Esterases in the Brain*

By means of the methods detailed above it was possible clearly to distinguish the following six types of esterase in brain tissue:

(1) *Acetylcholinesterase (AChE)*

Acetylcholinesterase was identified by its ability to hydrolyse AThCh in the presence of the specific pseudocholinesterase inhibitor *iso*-OMPA. The possibility that other esterases besides AChE could hydrolyse AThCh was controlled in each instance by using the specific AChE inhibitor 62.C.47 in low molar concentrations and noting complete inhibition. In this way acetylcholinesterase was found to have its highest activity in the caudate nucleus. Because of the absence of significant concentrations of other hydrolytic enzymes in this nucleus, the enzyme was demonstrated equally well here by the indoxyl method. When used in conjunction with 62.C.47, the indoxyl method showed approximately 90% inactivation of enzymic activity in this nucleus. In other places showing high acetylcholinesterase activity, however, such as the neurones of the hypothalamic nuclei, the high concentration of the other esterases entirely obscured the effect of the inhibitors with the indoxyl substrate and demonstration of AChE had to be done by using AThCh. In the cortex,

TABLE 2.—THE EFFECT OF DIFFERENT CONCENTRATIONS OF INHIBITORS ON THE BRAIN ESTERASES

Inhibitors	Pericyte	Ch. Plexus	Neurone	Pseudo ChE	Specific ChE
E600 10^{-3} M	−4	−4	−4	−4	−4
E600 10^{-4} M	N	−2	−4	−4	−4
E600 10^{-5} M	N	−1	−4	−4	−4
E600 10^{-6} M	N	−1	−1	−4	−4
p-Chloro MPSA 10^{-3} M	N	N	N	−3	N
p-Chloro MPSA 10^{-4} M	N	N	N	−2	N
p-Chloro MPSA 10^{-5} M	N	N	N	N	N
p-Chloro MPSA 10^{-6} M	N	N	N	N	N
Eserine 10^{-4} M	N	N	N	−4	−4
Eserine 10^{-5} M	N	N	N	−4	−4
Eserine 10^{-6} M	N	N	N	−2	−4
62.C.47 10^{-3} M	N	N	N	N	−4
62.C.47 10^{-4} M	N	N	N	N	−4
62.C.47 10^{-5} M	N	N	N	N	−4
62.C.47 10^{-6} M	N	N	N	N	−2
SDS 10^{-2} M	N	N	N	−4	−4
SDS 10^{-3} M	N	N	N	−4	−4
SDS 10^{-4} M	N	N	N	N	N
SDS 10^{-5} M	N	N	N	N	N
$AgNO_3$ 10^{-2} M	−2	−3	−4	−4	N
$AgNO_3$ 10^{-3} M	N	N	−4	−4	N
$AgNO_3$ 10^{-4} M	N	N	−2	−4	N
$AgNO_3$ 10^{-5} M	N	N	N	−2	N
$AgNO_3$ 10^{-6} M	N	N	N	N	N
Mipafox 10^{-3} M	N	−2	N	−4	−4
Mipafox 10^{-4} M	N	−2	N	−2	−4
Mipafox 10^{-5} M	N	N	N	N	N
Mipafox 10^{-6} M	N	N	N	N	N
Hydroxylamine 10^{-3} M	N	N	N	−3	−3
Hydroxylamine 10^{-4} M	N	N	N	−2	−2
Hydroxylamine 10^{-5} M	N	N	N	N	N
Hydroxylamine 10^{-6} M	N	N	N	N	N
iso-OMPA 10^{-3} M	N	N	N	−4	N
iso-OMPA 10^{-4} M	N	N	N	−4	N
iso-OMPA 10^{-5} M	N	N	N	−4	N
iso-OMPA 10^{-6} M	N	N	N	−4	N

VOL 1 1956/

TABLE 3.—THE EFFECT OF pH ON THE HYDROLYSIS OF O-ACETYL-5-BROMOINDOXYL BY BRAIN ESTERASES

pH before incubation	Pericyte	Choroid Pl.	Neurone	Pseudo ChE	Specific ChE	pH after incubation
2·1	−2	−3(−4)	−4	−4	−4	3·4
2·4	−2	−3(−4)	−4	−4	−4	3·8
3·0	N	−3	−3	−4	−4	4·0
3·8	N	−2	−3	−4	−4	4·2
4·6	N	−1	−2	−3	−3	4·8
4·9	N	−1	−2	−3	−3	5·1
5·1	N	N	−1	−2	−2	5·3
5·3	N	N	N	−2	−2	5·5
5·6	N	N	N	−1	−1	5·6
5·8	N	N	N	−1	−1	5·9
6·2	+1	+1	+1	+1	+1	6·2
7·0	N	N	N	N	N	6·8
8·0	N	N	N	N	N	7·6

VOL. 1 56/57

significant enzyme activity was shown only in occasional neurones, although the fibres as a rule were fairly heavily stained. Scattered neurones were positive throughout the nuclei in the sections examined. In general, the distribution of this enzyme using the present methods agrees with that of KOELLE (1954).

(2) *Pseudocholinesterase* (*non-specific ChE*)

This enzyme was recognized by the fact that it hydrolyses AThCh, BuThCh, and 5BrI and that it can be completely inhibited by low molar concentrations of *iso*-OMPA It is totally insensitive to the specific acetylcholinesterase inhibitor 62.C.47. Like the specific cholinesterase, this enzyme was also completely inhibited by eserine (10^{-4} M), but it appeared to be less sensitive to Mipafox (10^{-4} M) and more sensitive to the mercurial inhibitor and to silver nitrate (Table 1). The optimum pH for hydrolysis of 5-BrI by pseudocholinesterase was in the region of 6·2 and in this respect no histochemical differentiation could be made between the two cholinesterases (Table 3).

Pseudocholinesterase was found in a high concentration in the neurones of the habenular nuclei, especially in the medial habenular nucleus (Figs. 2 and 3). The cerebral capillaries contained fairly large amounts of the enzyme, and it is also found diffusely distributed throughout the neuropile with a slightly higher concentration in the white than in the grey matter, suggesting some relationship with the myelin sheaths. The smooth muscle fibres in the larger blood vessels also contained large amounts of this enzyme. Using BuThCh as substrate, KOELLE (1954) found pseudocholinesterase activity only in the neurones showing high AChE content and therefore questioned the specificity of the substrate. It should be noted that with the aid of the inhibitors used in this study the pseudocholinesterase distribution was shown to be completely independent of that of the specific cholinesterase. Moreover, it was not possible with any of the methods used to demonstrate any cholinesterase in the fibrous astrocytes (cf. KOELLE, 1954).

(3) *The "non-specific esterases"*

(i) *The "non-specific esterase" of most neurones of the cerebral cortex (type* I). This enzyme and the ones to be discussed below (types II, III and IV) were differentiated from the cholinesterases by the fact that when using the indoxyl substrate the cells containing them gave a normal staining reaction after inhibition with eserine (10^{-4} M), (10^{-4} M), *iso*OMPA (10^{-4} M), hydroxylamine (10^{-3} M), and sodium dodecyl sulphate (10^{-3} M). Like the cholinesterases, these esterases had their optimum pH at $\pm 6{\cdot}2$ and they were activated by hydrocinnamic acid (10^{-3} M) and by cysteine (10^{-3} M). Type-I esterase could be differentiated from types II, III, and IV by means of its greater sensitivity to E600 (10^{-5} M) and $AgNO_3$ (2×10^{-3} M) (Tables 1 and 2). Lowering the pH of the incubation mixture (Table 3) served as further evidence for distinguishing type I from type II.

(ii) *The "non-specific esterase" of the pericytes and of the neurones of the hypothalamic nuclei (type* II). This enzyme was especially prominent in the pericytes and in the large neurones of the supraoptic, paraventricular, mamillary, and premamillary nuclei. It was resistant to all the inhibitors tested except silver nitrate at 2×10^{-3} M and E600 at 10^{-3} M. It could further be differentiated from the other enzymes in that at pH 3 it still showed almost maximal activity (Table 3). It was active in the entire pH range tested, viz. pH 2·1–8.

(iii) *The "non-specific esterase" of the choroid plexus (type* III). This enzyme was essentially similar to type II except in its sensitivity to Mipafox (10^{-4} M) and in that its activity was to some extent inhibited pH 4·9.

(iv) *The "non-specific esterase" of the ependyma of the third ventricle (type* IV). This enzyme was differentiated from the others mainly because it had its pH optimum at 4·6. In other respects it behaved like type II. A possible functionally significant finding is the constantly increased amount of type IV esterase in the ependyma lying opposite some of the hypothalamic nuclei, when this is compared with ependyma in other sites. The esterase of the subcommissural organ appears to be of type IV.

Fig. 1 shows the overall picture obtained on the right with 5BrI and on the left with AThCh as substrates, in coronal sections of rat brain.

II. *Functional Changes in the Esterases of the Hypothalamic Nuclei*

The following experiments were designed to investigate changes in the two enzymes detected in the hypothalamic nuclei in various conditions:

(1) *Saline-treated rats*

A group of six rats (± 200 g) were given a 2·5% solution of sodium chloride in place of ordinary drinking-water in order to overload the anti-diuretic function of the posterior lobe of the pituitary gland. Two rats were killed on the eighth, ninth, and tenth days of the experiment and their brains examined in the way described.

(2) *Lactating rats*

Four rats were used for this investigation. The rats were killed daily from the thirteenth to the sixteenth day of lactation.

VOL 1 1956/

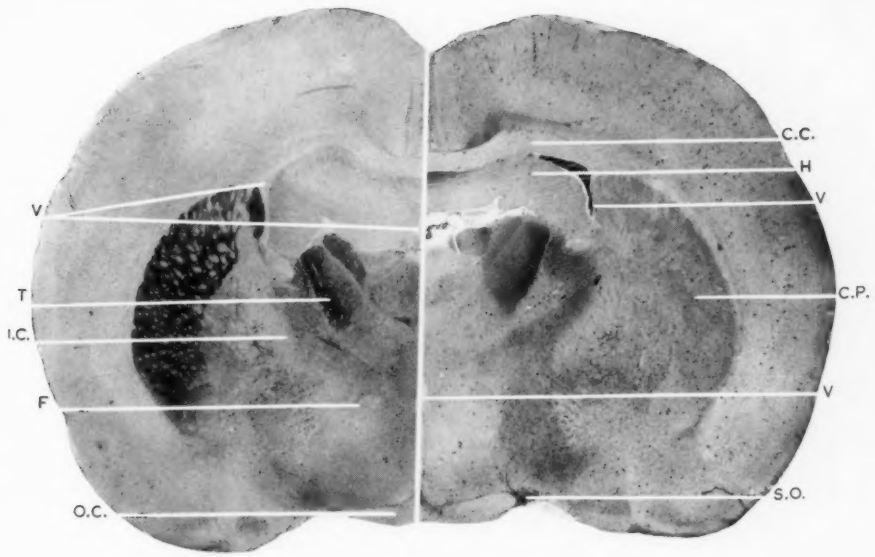


FIG. 1.—Left side: rat brain incubated with AThCh for 6 hours.
Right side: rat brain incubated with 5-BrI for 1½ hours.
C.C: Corpus Callosum. H: Hippocampus. C.P: Caudate-Putamen complex. F: Fornix.
I.C: Internal Capsule. O.C: Optic Chiasma. V: Ventricle. T: Thalamus.
S.O: Supraoptic nucleus.

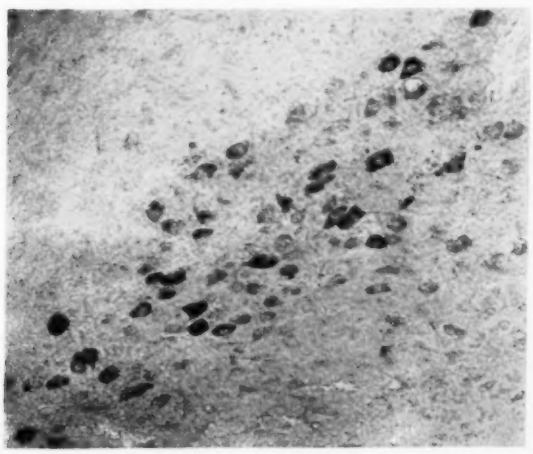


FIG. 2.—Medial habenular nucleus showing pseudocholinesterase in the neurones after incubation with AThCh + 62.C.47. ×150

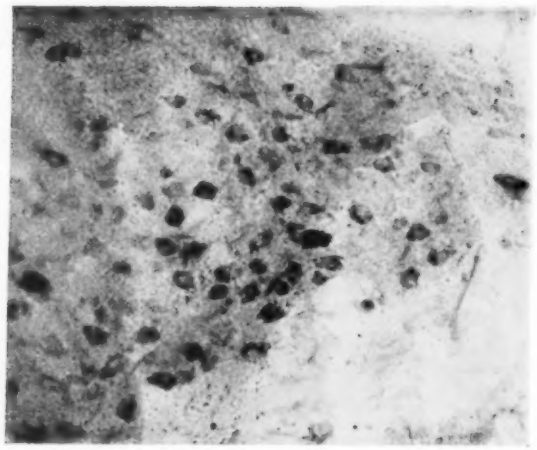


FIG. 3.—Medial habenular nucleus showing pseudocholinesterase reaction after incubation with BuThCh. ×150

VOL
1
1956/

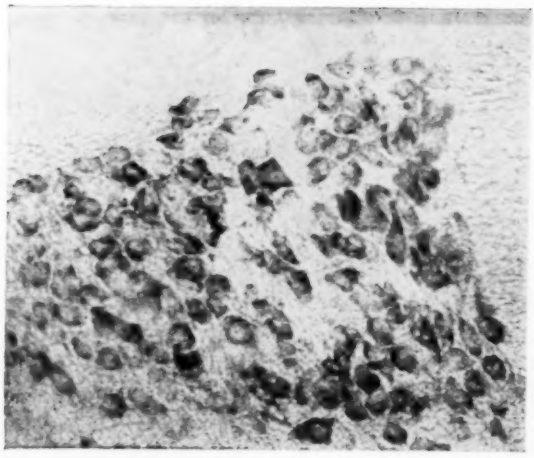


FIG. 4.—Supraoptic nucleus in control rat demonstrating AChE in the neurones. AThCh. ×205

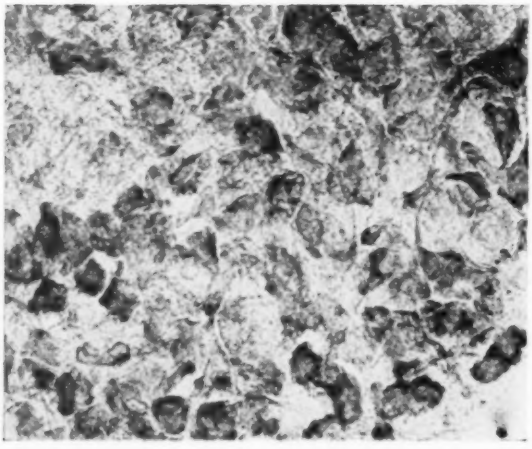


FIG. 5.—Supraoptic nucleus in a saline-treated rat killed on the ninth day. There is hypertrophy of the cells associated with an increase in the amount of AChE. AThCh. ×205

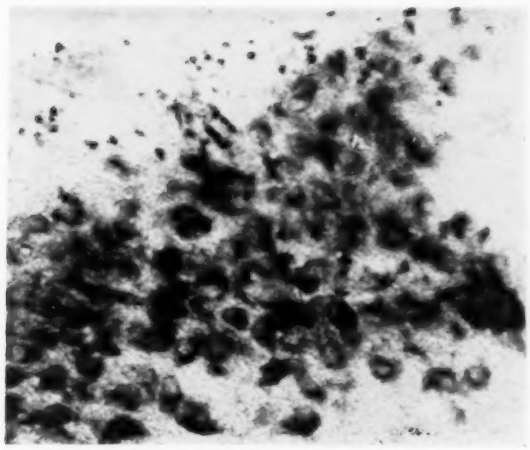


FIG. 6.—Supraoptic nucleus in control rat. Section incubated in 5-BrI for 1½ hours. Note the indoxyl esterase in the neurones and in the elongated very heavily staining pericytes. ×205

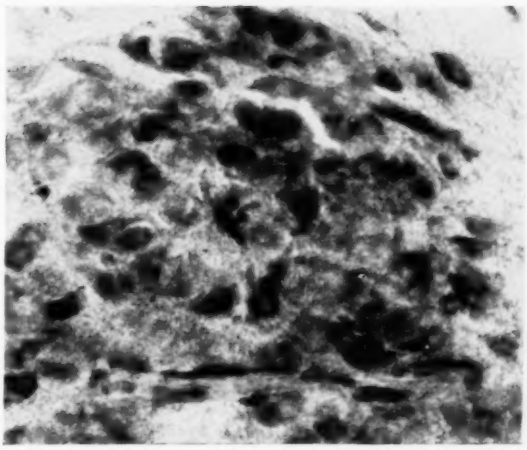


FIG. 7.—Supraoptic nucleus in a saline-treated rat showing a decrease of the indoxyl esterase in the hypertrophied cells. The majority of dark cells are pericytes. 5-BrI. ×205

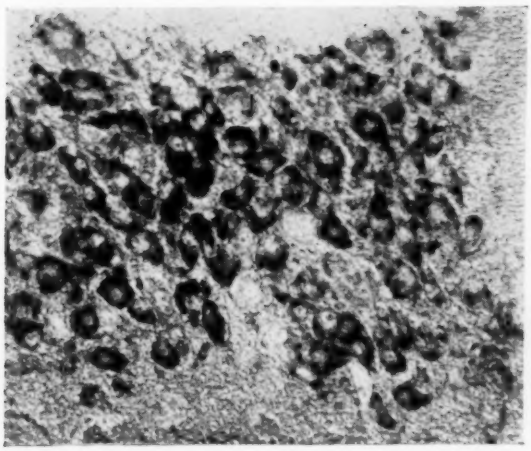


FIG. 8.—Lactating rat. The supraoptic nucleus shows essentially similar but less pronounced changes to those depicted in Fig. 5. AThCh. ×205

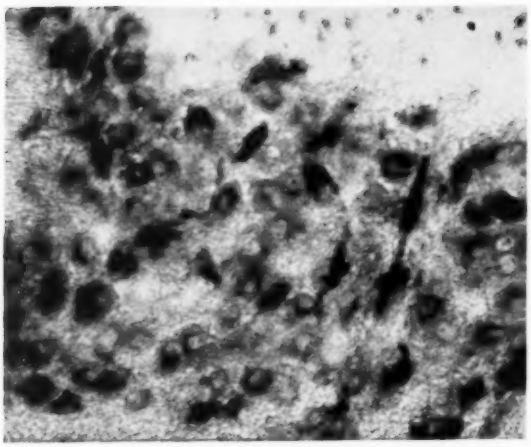


FIG. 9.—Supraoptic nucleus of a lactating rat demonstrating hypertrophy of the neurones and decreased amounts of indoxyl esterase. Many of the dark cells are pericytes but some are neurones. 5-BrI. ×205

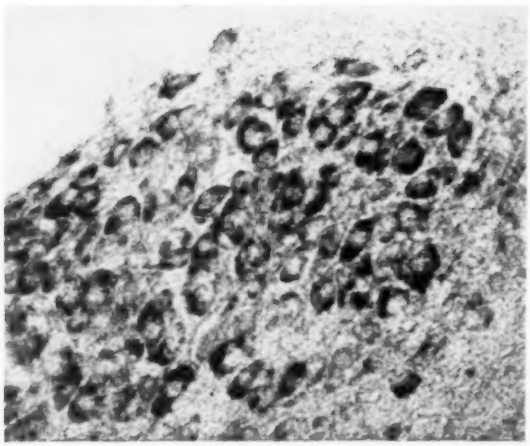


FIG. 10.—Castrated rat. There is an increase in the acetylcholinesterase of the hypertrophied supraoptic neurones. AThCh. ×205

VOL
1
1956/

(3) *Castrated rats*

Five rats were castrated under ether anaesthesia and subsequently killed from 26 to 30 days after the operation.

(4) *Thiouracil-treated rats*

A group of six rats were given a saturated solution of 4-methyl-2-thiouracil in place of the ordinary drinking-water and killed on the tenth to fifteenth day after starting the experiment.

Control animals of approximately the same weights and ages were killed and examined in conjunction with every experimental animal.

III. *Results and their Significance*

In the saline-treated rats, serial sections of the hypothalamic area showed marked hypertrophy of the cells of the supraoptic, paraventricular, and filiform nuclei. There was a suggestion that some of the cells of the anterior hypothalamic nucleus were also hypertrophic, but because of the ill-defined nature of this nucleus, accurate comparison with the normal was impossible. Associated with this hypertrophy there was a significant increase of AChE, as shown with the KOELLE method (Figs. 4 and 5). The indoxyl esterase, however, showed a distinct decrease at this time (Figs. 6 and 7). HILLARP (1949) described chromatolytic changes in the supraoptic and paraventricular nuclei of rats 6 hours after a subcutaneous injection of 10 ml of a 5% saline solution. Three rats were treated in exactly this way, but no change could be demonstrated in the esterase reaction of these nuclei. It appears therefore that overloading of the antidiuretic function has to be present for some time before histochemical changes in esterase levels can be detected in the hypothalamic nuclei.

No demonstrable changes could be detected in any of the other hypothalamic nuclei in either group of rats.

In the group of lactating rats, sections of the hypothalamic area showed similar but less obvious morphological and histochemical changes as the saline rats, viz. hypertrophy of the cells associated with increased acetylcholinesterase and decreased indoxyl esterase (Figs. 8 and 9).

The castrated rats showed essentially similar, though much less pronounced, changes in the supra-optic nucleus than those in the previous two groups (Fig. 10), No changes could be seen in the other nuclei, but it should be noted that unless the morphological and histochemical changes are severe, they are very likely to be missed.

In the group of rats treated with thiouracil no significant histochemical or morphological changes could be seen in any of the hypothalamic nuclei.

It is generally accepted that the hypothalamic mechanisms regulating the activity of the posterior pituitary are in outline very similar to those regulating the anterior pituitary, and it has been suggested that the chemical transmitter responsible for stimulation of the hypothalamic cells is acetylcholine (PICKFORD, 1947; DUKE, PICKFORD, and WATT, 1950; ABRAHAMS and PICKFORD, 1956). In the experiments described above there is an increased demand for both the posterior lobe hormones (antidiuretic and oxytocic) and the anterior lobe hormones (gonadotrophic, lactogenic, and thyrotrophic) of the pituitary gland. Under these conditions one would expect an increased metabolism of acetylcholine and an associated increase of acetylcholinesterase necessary for the breakdown of free acetylcholine. Except in the

group of thiouracil-treated rats, it is possible to show an increased amount of acetylcholinesterase in the supraoptic, paraventricular, and filiform nuclei, and this observation lends further support to the hypothesis that acetylcholine stimulates the supraoptic and possibly other hypothalamic cells.

The *in vivo* function of the indoxyl esterase and its exact nature (? esterase, ? peptidase) are as yet undecided. In considering the changes observed with this enzyme, two possibilities have to be considered before attaching to it any functional significance under the experimental conditions studied. Firstly, the decrease may be a relative one, that is, an apparent decrease due to hypertrophy of the cells. Secondly, it may be an absolute decrease, and thus of definite functional importance. It is, however, impossible to assess the true significance of this finding at present.

DISCUSSION

Owing to the fact that the halogen-substituted indoxyl substrates are hydrolysed by non-specific esterases, lipases, specific cholinesterases, non-specific cholinesterases, and possibly other hydrolytic enzymes, and because of the multiplicity of these enzymes in a given cell or area, it is necessary, even with the aid of inhibitors, to use other substrates in combination with 5-BrI. Initially BuThCh was used in addition to AThCh for studying the cholinesterases, but it was soon found that AThCh, in combination with inhibitors, gave equally good results with pseudocholinesterase. The use of BuThCh was therefore discontinued. This finding was confirmed in a preliminary study of other tissues of the rat. It was also found that, except at sites of high cholinesterase activity, the KOELLE method was more reliable and sensitive than the indoxyl method as an indicator for these enzymes.

It has been shown by ORD and THOMPSON (1952) and by BAYLISS and TODRICK (1956) that AChE is partly responsible for the hydrolysis of BuThCh by homogenates of rat brain. The latter authors have therefore suggested that the biochemical determination of AChE in the presence of excess pseudocholinesterase, or *vice versa*, could be improved by using selective inhibitors. Two of the inhibitors used for this purpose were 62.C.47 (BURGEN, 1948) and *iso*-OMPA (ALDRIGE, 1953). 62.C.47 is five hundred times as effective against AChE as against pseudocholinesterase and *iso*-OMPA is stated to be at least five hundred times as effective against pseudocholinesterase as against AChE. In the present investigation these two inhibitors were applied histochemically with excellent results (Tables 1 and 2). As suggested above, the use of *iso*-OMPA eliminated the need to use BuThCh as substrate.

With regard to the cholinesterases of the rat brain, it was further found that sodium dodecyl sulphate produced total inhibition at a concentration of 10^{-3} M and that hydroxylamine hydrochloride (10^{-3} M) produced $\pm 90\%$ inhibition (Tables 1 and 2). Silver nitrate (2×10^{-3} M) produced complete inhibition of the pseudocholinesterase when using 5-BrI as substrate, but left the acetylcholinesterases intact.

It is usually stated that 10^{-4} M E600 inhibits non-specific esterases completely (MYERS, SCHOTTE, and MENDELL, 1955), but, in spite of using the inhibitor at this concentration, the 5-BrI substrate was still being hydrolysed by enzymes in various sites in the rat brain (Table 1). Furthermore, the fact that these enzymes were also resistant to 10^{-4} M eserine eliminated the possibility of their being cholinesterases (DENZ, 1953). In view of these findings and the fact that the indoxyl substrates (O-acetylindoxyl, O-acetyl-5-bromoindoxyl) are hydrolysed by trypsin and

chymotrypsin (PEARSE, 1956), the possibility of their being proteolytic enzymes was considered. Although trypsin and chymotrypsin are sensitive to 10^{-4} M E600, other proteolytic enzymes are known to be resistant (MYERS, SCHOTTE, BOER, and BORSJE-BAKKER, 1955).

In order to try to solve this problem histochemically, the following inhibitors were used in addition to those usually employed in the identification of esterases. (1) Hydrocinnamic acid as inhibitor for carboxypeptidase (ELKINS-KAUFMAN and NEURATH, 1949); (2) Hydroxylamine hydrochloride as inhibitor for aminopeptidase (SCHALES and HILL, 1949); (3) Silver nitrate as inhibitor for aminopeptidase, dipeptidase, and prolinase (ABDERHALDEN and MERKEL, 1936); (4) Cysteine was used primarily because it is a known activator for cathepsins. The results that were obtained with these inhibitors (Table 1), viz. inhibition of the pericyte, neurone, and choroid plexus enzymes with silver nitrate, their activation with cysteine, and the resistance of these enzymes to E600 and eserine, strongly suggest that they are peptidases. The better results obtained with silver nitrate can possibly be explained on the basis of its wider power of inhibition. The very low pH at which these enzymes are still functioning and the fact that the optimum pH of the ependymal esterase proved to be approx. 4·6 is still further evidence in favour of the peptidase nature of these enzymes. ANSELL and RICHTER (1954) found the optimum pH of brain cathepsin to be 3·5 to 3·8.

Repeated claims have been made that glial cells, and especially the fibrous astrocytes, contain pseudocholinesterase (KOELLE, 1950; KOELLE, 1954; CAVANAGH, THOMPSON, and WEBSTER, 1954). In the rat we were unable to demonstrate any enzyme activity in the astrocytes with the substrates employed. The same substrates were also employed in the study of an astrocytoma in fresh human autopsy material, with negative results.

SUMMARY

(1) The esterases of the rat brain have been studied histochemically by employing O-acetyl-5-bromoindoxyl and various other substrates. By using different inhibitors and by changing the pH of the incubation mixture, it has been possible to identify at least six types of enzymes in different locations.

(2) These have been divided into specific and non-specific (pseudo) cholinesterases and into four non-specific esterases.

(3) On the basis of our findings, it is suggested that some of the latter may be identical with the intracellular peptidases.

(4) The two enzymes demonstrated histochemically in the hypothalamic nuclei have been studied under various experimental conditions. It has been shown that in conditions known to stimulate either the anterior or the posterior lobe of the pituitary there is an increase in the acetylcholinesterase content of some of the hypothalamic nuclei.

(5) This suggests that the hormones of both these lobes of the pituitary can be released by cholinergic transmission to some of the hypothalamic neurones.

Acknowledgements—We are grateful to Dr. W. N. ALDRIDGE for supplying us with E600 and to Dr. PRESCOTT of the Wellcome Foundation Ltd. who kindly provided us with samples of 62.C.47.

REFERENCES

ABDERHALDEN E. and MERKEL R. (1936) *Fermentforschung* **15,** 1.
ABRAHAMS V. C. and PICKFORD M. (1956) *J. Physiol.* **133,** 330.
ALDRIDGE W. N. (1953) *Biochem. J.* **53,** 62.
ANSELL G. B. and RICHTER D. (1954) *Biochim. Biophys. Acta* **13,** 87.
BARRNETT R. J. (1952) *Anat. Rec.* **114,** 577.
BARRNETT R. J. and SELIGMAN A. M. (1953) *Science* **114,** 579.
BAYLISS B. J. and TODRICK A. (1956) *Biochem. J.* **62,** 62.
BURGEN A. S. V. (1948) *Brit. J. Pharmacol.* **4,** 219.
CAVANAGH J. B., THOMPSON R. H. S., and WEBSTER G. R. (1954) *Quart. J. exp. Physiol.* **39,** 185.
DENZ F. (1953) *Brit. J. exp. Path.* **34,** 329.
DUKE H. N., PICKFORD M., and WATT J. A. (1950) *J. Physiol.* **111,** 81.
ELKINS-KAUFMAN E. and NEURATH H. (1949) *J. biol. Chem.* **178,** 645.
GOMORI G. (1948) *Proc. Soc. exp. Biol., N.Y.* **68,** 354.
GOMORI G. (1952) *Microscopic Histochemistry—Principles and Practice* Univ. of Chicago Press, Chicago.
GOMORI G. and CHESSICK R. D. (1953) *J. Neuropath. exp. Neurol.* **12,** 387.
HILLARP N. A. (1949) *Acta Endocrinol.* **2,** 33.
KOELLE G. B. (1950) *J. Pharmacol.* **100,** 158.
KOELLE G. B. (1951) *J. Pharmacol.* **103,** 153.
KOELLE G. B. (1954) *J. comp. Neurol.* **100,** 211.
KOELLE G. B. (1955) *J. Neuropath. exp. Neurol.* **14,** 23.
KOELLE G. B. and FRIEDENWALD J. S. (1949) *Proc. Soc. exp. Biol., N.Y.* **70,** 617.
MALATY H. A. and BOURNE G. H. (1955) *Acta Anat.* **23,** 337.
MYERS D. K., SCHOTTE A., BOER H., and BORSJE-BAKKER H. (1955) *Biochem. J.* **60,** 481.
ORD M. G. and THOMPSON R. H. S. (1952) *Biochem. J.* **51,** 245.
PEARSE A. G. E. (1953) *Histochemistry: Theoretical and Applied.* Churchill, London.
PEARSE A. G. E. (1956) *J. Path. Bact.* (in press)
PICKFORD M. (1947) *J. Physiol.* **106,** 264.
RAVIN H. A., ZACKS S., and SELIGMAN A. M. (1953) *J. Pharmacol.* **107,** 37.
SCHALES O. and HILL B. R. (1949) *Arch. Biochem.* **22,** 366.

VOL
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 203-206. Pergamon Press Ltd., London

ÜBER DIE GANGLIOSIDSPEICHERUNG IM NERVENGEWEBE BEI DER INFANTILEN AMAUROTISCHEN IDIOTIE VOM TYP TAY-SACHS UND DIE BEI DER KONSERVIERUNG DES MATERIALS IN FORMALIN AUFTRETENDEN VERÄNDERUNGEN

THE STORAGE OF GANGLIOSIDES IN NERVOUS TISSUE IN TAY-SACHS DISEASE AND THE CHANGES IN MATERIAL PRESERVED IN FORMALIN

E. KLENK, W. VATER, und G. BARTSCH
Aus dem physiologisch-chemischen Institut der Universität Köln

(*Received* 12 *October* 1956)

DIE infantile amaurotische Idiotie vom Typ Tay-Sachs gehört zusammen mit der Niemann-Pick'schen Krankheit zu denjenigen Neurolipoidosen, über welche bereits eingehendere chemische Untersuchungen (siehe dazu KLENK, 1955) vorliegen. Beide Krankheiten gleichen sich in ihrem histopathologischen Erscheinungsbild weitgehend (LETTERER, 1939). Dass sie in ihrem Wesen nahe miteinander verwandt sind, ergibt sich auch auf Grund der chemischen Befunde, aus welchen hervorgeht, dass in beiden Fällen vorzugsweise Sphingolipoide gespeichert werden. Trotz der unverkennbaren Ähnlichkeit kann aber auch kein Zweifel darüber bestehen, dass die Speichersubstanzen bei beiden Krankheiten nicht identisch sind. Während bei der Niemann-Pick'schen Krankheit Sphingomyelin (KLENK, 1935) die eigentliche Speichersubstanz ist, handelt es sich bei der Tay-Sachs'schen Krankheit um Ganglioside (KLENK, 1939; 1942). Auf das unterschiedliche Verhalten bei der vergleichend histologischen Untersuchung hat besonders FEYRTER (1939) hingewiesen.

Im Folgenden berichten wir über die Untersuchungsergebnisse von einem weiteren Tay-Sachs-Fall, welcher an der Bonner Universitäts-Kinderklinik (Direktor: Prof. Dr. ULLRICH) im Jahre 1949 beobachtet und VON BREHM (1949) beschrieben wurde. Es stand eine ganz frische, möglichst kurz post mortem entnommene, etwa 2·5 cm dicke Gehirnscheibe, ein Frontalschnitt auf der Höhe des Chiasma opt., zur Verfügung. Für die Gangliosidbestimmung wurden kleinere Proben herauspräpariert, die man auf ein Uhrglas in dünner Schicht ausstrich und im Vakuumexsikkator über Schwefelsäure scharf trocknete. Wie in allen bisher von uns untersuchten Fällen dieser Art fand sich in der grauen Substanz des Nervengewebes wieder eine starke Gangliosidanreicherung, die im Cortex des Cerebums den höchsten Betrag und zwar über das 10-fache des Normalwerts erreichte. Die Tabelle 1 enthält die

in der Gangliosidfraktion nach KLENK u. LANGERBEINS (1941) bestimmten Neuraminsäure(NS)-Werte, aus welchen man den Gangliosidgehalt durch Multiplikation mit dem Faktor 5 (NS-Gehalt der Ganglioside 20%) erhält.

TAB. 1.—NEURAMINSÄUREGEHALT DES GEHIRNS (FRISCHES, UNFIXIERTES MATERIAL) BEI DER TAY-SACHS'SCHEN KRANKHEIT IN % DES TROCKENGEWEBES

Cortex (Cerebrum)	1·52–1·64†	Nucl. caud.	1·0
Nucl. ruber u. niger	1·2	Putamen	1·0
Thalamus	1·1	Pallidum	1·0

† Normal 0·12–0·13.

In der weissen Substanz war der NS-Gehalt wesentlich niedriger. Bei allen derartigen Proben wurde anstelle der charakteristischen schönen Violettfärbung mit Bials Reagens eine uncharakteristische Braunfärbung erhalten.

Eine andere Scheibe desselben Gehirns wurde in Formalin aufbewahrt. Nach Ablauf von 6 Jahren wurden in dem formolfixierten Material wieder der Gangliosidgehalt bestimmt. In der Cortex waren jetzt die NS-Werte auf 1·09–1·12% abgesunken. Da dieser Befund darauf schliessen liess, dass bei der schwach sauren Reaktion (pH = 5) des Konservierungsmittels die Neuraminsäure im Lauf der Zeit hydrolytisch von den Gangliosiden abgespalten und aus dem Material herausgelöst wird, haben wir den Versuch gemacht, die Veränderung auch auf präparativem Wege durch Isolierung der nach Abspaltung der Neuraminsäure verbleibenden Glykolipoide festzustellen.

Zu diesem Zweck wurde aus dem gut gewässerten Gewebestück die Cortex abpräpariert und das Material der Reihe nach mit Aceton, Äther und heissem Methanol-Chloroform (1 : 1 V.T.) extrahiert. Jedes der so gewonnenen Lipoidpräparate nahm man in Äther auf, zentrifugierte die ätherunlösliche Protagonfraktion ab und fällte aus der stark eingeengten ätherischen Lösung die Glycerinphosphatide mit Aceton aus. Der in Lösung bleibende Teil bestand der Hauptsache

TAB. 2.—LIPOIDFRAKTIONEN DER CORTEX NACH 6-JÄHRIGER AUFBEWAHRUNG DES MATERIALS IN FORMALIN

	Menge (g)	% P*	% Glycerin†	% Zucker‡ ber. als Galaktose	% Hexosamin§	% NS
Ausgangsmaterial(trocken)	1·72					
Cholesterin und Fettsäuren	0·16	0·04 (0·05)	—	—	0·4 (0·5)	—
Glycerinphosphatide	0·02	—	—	—	1·9 (2.1)	—
Protagonfraktion	0·28	2·28 (2·32)	5·2 (5·9)	24·2 (24·9)	6·2 (6·2)	5·9 (6·0)

* Nach TEORELL (1931).
† Nach BLIX (1938).
‡ Spaltung mit 10% iger HCl und Zucker nach SOMOGYI (1937) bestimmt
§ Nach BLIX (1948).

VOL 1 1956/

nach aus Cholesterin und Fettsäuren. Die entsprechenden Fraktionen wurden vereinigt. Das Ergebnis ist in der Tab. 2 zusammengestellt.

Die Protagonfraktion löste man nun zur Abtrennung der darin in reichlichen Mengen vorhandenen Phosphatide (0·16 g*); davon 0·13 g Glycerinphosphatide†) und 0·03 g Sphingomyelin) in der 5-fachen Menge heissen Pyridins. Nach 12 tägigem Stehen saugte man die auskristallisierten Phosphatide ab, engte die Lösung im Vakuum ein und fällte die gelöst gebliebenen Glykolipoide mit Aceton aus. Die im Vakuum über Schwefelsäure getrocknete Substanz löste man in einem Gemisch von 6 ccm Chloroform, 4 ccm Methanol, 2 ccm Äthanol und entmischte durch Zufügen von 4 ccm Wasser. Nach kräftigem Schütteln im Scheidetrichter trennte man die beim Stehen sich absetzende untere Chloroform-Phase von der oberen wässrig-alkoholischen Schicht ab und unterwarf die aus der Chloroform-Phase durch Abdampfen des Lösungsmittels zurückgewonnene Substanz noch zweimal demselben Trennungsverfahren. Aus den vereinigten wässerig-alkoholischen Phasen wurde die Gangliosidfraktion(I) durch Gefriertrocknung und aus der Chloroform-Phase die NS-freie Glykolipoidfraktion(II) gewonnen. 200 mg der Protagonfraktion lieferten auf diese Weise 80 mg Substanz I und 95 mg Substanz II. Die analytischen Daten sind in Tab. 3 zusammengestellt.

TAB. 3.—DIE ZUSAMMENSETZUNG DER GLYKOLIPOIDFRAKTIONEN DER CORTEX NACH 6-JÄHRIGER AUFBEWAHRUNG DES MATERIALS IN FORMALIN

	% P	% Zucker ber. als Galaktose	% Hexosamin	% NS
I	0·74 (0·77)	30·9 (31·3)	15·0 (15·3)	11·2 (11·6)
II	1·4 (1·6)	18·9 (19·1)	10·3 (10·8)	neg.

Wenn es infolge der kleinen Substanzmengen auch nicht möglich war, aus den Präparaten die Phosphatide vollständig zu entfernen, so ergibt sich aus den Versuchen doch eindeutig, dass ausser den Gangliosiden noch andere zurckerreiche Glykolipoide vorhanden sind, welche ebenso wie die Ganglioside Hexosamin als Baustein enthalten, sich aber von den Gangliosiden durch das Fehlen von NS unterscheiden. Glykolipoide dieser Art, die ihrer chemischen Natur nach eine Mittelstellung einnehmen zwischen den Gangliosiden und den einfachen Cerebrosiden, sind aus dem Erythrocytenstroma verschiedener Tiere gewonnen worden (KLENK u. LAUENSTEIN, 1951, 1952, u. 1953). Wenn man berücksichtigt, dass die Substanz II auf Grund des P-Werts noch etwa 30% Phosphatide enthält, so muss dem darin als Hauptbestandteil vorhandenen NS-freien Glykolipoid ein Zuckergehalt von etwa 30% (darunter 15% Hexosamin) zukommen. Es spricht alles dafür, dass es sich hier im wesentlichen um sekundär gebildete Produkte handelt, die beim langen Aufbewahren in Formalin aus den Gangliosiden durch Abspaltung der NS entstanden sind. Da bei der grossen Seltenheit dieser Krankheit sowohl die chemischen, wie auch die histologischen Untersuchungen vielfach mit älteren formolfixierten Organstücken ausgeführt werden, wird man bei der Auswertung der Befunde den

* ber. aus dem P-Wert.
† ber. aus dem Glyceringehalt.

hier festgestellten Veränderungen der Natur des gespeicherten Lipoids Rechnung tragen müssen.

ZUSAMMENFASSUNG

Bei einem weiteren Fall von infantiler amaurotischer Idiotie vom Typ Tay-Sachs fand sich wie bei allen früher untersuchten Fällen eine starke Gangliosidanreicherung in der grauen Substanz des Gehirns. Bei der längeren Aufbewahrung der Gehirnstücke in Formalin nimmt der Gangliosidgehalt ab, wobei neuraminsäurefreie Glykolipoide von relativ hohem Hexosamingehalt entstehen.

SUMMARY

In a case of infantile amaurotic familial idiocy (type Tay-Sachs) there was a great increase in the concentration of gangliosides in the grey matter of the brain. The increase was similar to that reported in other cases. On keeping the pieces of brain in formaldehyde for a long time, the ganglioside content decreased and glycolipides with a rather high content of aminosugars, but free of neuraminic acid, were formed.

LITERATUR

BLIX G. (1938) *Mikrochim. Acta* **1,** 75.

BLIX G. (1948) *Acta Chim. Skand.* **2,** 467.

BREHM R. (1949) Dissertation, Bonn.

FEYRTER F. (1939) *Virchows Arch.* **304,** 481.

KLENK E. (1935) *Z. physiol. Chem.* **255,** 24.

KLENK E. (1939) *Z. physiol. Chem.* **262,** 128.

KLENK E. (1942) *Ber. dtsch. Chem. Gesellsch.* **75,** 1632.

KLENK E. (1955) in H. WAELSCH *Biochemistry of the Developing Nervous System* p. 397. Acad. Press, New York.

KLENK E. u. LANGERBEINS H. (1941) *Z. physiol. Chem.* **270,** 185.

KLENK E. u. LAUENSTEIN K. (1951) *Z. physiol. Chem.* **288,** 220.

KLENK E. u. LAUENSTEIN K. (1952) *Z. physiol. Chem.* **291,** 249.

KLENK E. u. LAUENSTEIN K. (1953) *Z. physiol. Chem.* **295,** 164.

LETTERER E. (1938) *Verh. d. ges. für Verdauungs-u. Stoffwechselkrnakheiten* XIV S. 41. Tagung in Stuttgart.

VOL.
1
1956/5

Journal of Neurochemistry, 1957, Vol. 1, pp. 207-215. Pergamon Press Ltd., London

CHOLESTEROL AND CEREBROSIDES IN BRAIN SPECIMENS PRESERVED FOR LONG PERIODS IN FORMALIN

R. RODNIGHT
Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation
University of London, Maudsley Hospital, London S.E.5

(*Received* 18 *January* 1957)

VOL. 1 56/57

CHOLESTEROL and the cerebrosides, together accounting for over 30% of the dry weight of adult white matter, appear chemically stable in formalin-preserved brain for periods as long as six months (BRANTE, 1949; CUMINGS, 1953). If this stability could be shown to extend for much longer periods, the opportunities for analytical work on the composition of the brain in rare diseases would be greatly increased, since many laboratories possess an accumulated stock of formalin-preserved brain specimens from a variety of conditions.

The object of the present study was to investigate this possibility. The stability of the two lipids has been examined in normal brain specimens preserved in formalin for several years and their content determined in a number of abnormal specimens, including material from three cases of schizophrenia, two of disseminated sclerosis, and four of gross mental retardation in childhood.

METHODS

"Analar" grade reagents or redistilled solvents were used throughout. The formalin employed was a 1:10 v/v mixture of saturated formaldehyde and tap-water.

Preparation of lipid extracts. Specimens were removed from the formalin and the superficial liquid blotted away with filter paper. Samples of grey and white matter were dissected out and washed lipid extracts prepared by the chloroform/methanol procedure of FOLCH, ASCOLI, LEES, MEATH, and LEBARON (1951). Whenever sufficient material was available, 3–4 g of grey matter and 1·5–2 g of white matter were taken and the final extract made up to 250 ml; smaller amounts were made up to a correspondingly smaller volume. Extracts were stored at −20°C pending analysis.

For cholesterol determination, aliquots of the extracts were treated as follows: solvents were removed at 60°C under reduced pressure; foaming in the terminal stages was controlled by the addition of ethanol. Cholesterol in the residue was separated from the phospholipids and taken up in a standard volume of acetone by the procedure of JOHNSON, MCNABB, and ROSSITER (1948).

Determination of cholesterol. Saponification of esterified cholesterol and separation of the resulting free cholesterol from the neutralized alkali was carried out by an adaptation of the method of SPERRY and BRAND (1943). Separation was found advisable, as the direct procedure of SPERRY and WEBB (1950) gave less satisfactory recoveries of added cholesterol.

Cholesterol was estimated in duplicate on 2 ml aliquots of the saponified and unsaponified acetone solutions according to SPERRY and WEBB (1950). For the final colour development at 25°C a large vacuum flask fitted with a test-tube rack served as a convenient light proof and portable water-bath. Colour intensity was determined in a Beckman D.U. spectrophotometer at 625 mμ.

Duplicate analyses rarely differed by more than 1% of their mean; on repeating the estimation on a new extract of the same tissue, variations of up to 5% of the mean were found.

Determination of cerebrosides. Four aliquots of each chloroform/methanol extract were taken, equivalent to 100–200 mg of grey matter or 50–100 mg of white matter, solvents removed as described

for the cholesterol determination and the lipid residue emulsified in 2 ml of water by alternate freezing and thawing. Two of the four samples were then heated with 2 ml of 6 N-HCl for 45 min on a boiling water-bath, neutralized, cleared, made up to 15 ml and filtered according to the method of BRAND and SPERRY (1941). The other two samples served as blanks and were treated by the same procedure in parallel, except that the 6 N-HCl was added after the period of heating. This simplified blank procedure gave the same result as the more elaborate one of BRAND and SPERRY (1941).

The hexose contained in 5 ml aliquots of the filtrate was estimated by the ferricyanide–ceric sulphate method of MILLER and VAN SLYKE (1936), using erio-green as indicator. After subtraction of blank values, the quantity of hexose found was converted to cerebroside by multiplying by 4·55; the factor was based on the assumption that all the reducing substance consisted of galactose.

Blank values with white matter extracts were usually about 10% of the total reducing substance; with grey matter extracts they ranged from 20% to 50% of the total. The variation between duplicates in a single determination was generally within 2% of their mean; on repeating the analysis in two instances, differences of 3% and 6% of the mean were found.

Significance of cerebrosides results. The values obtained by the method of BRAND and SPERRY (1941) include the hexose released by acid hydrolysis from other cerebroside and carbohydrate-containing lipids, as well as from the free cerebrosides ("free cerebrosides" include the galactosides or glucosides of ceramides—see THANNHAUSER and SCHMIDT, 1946). This observation applies particularly to grey matter, where SLOANE-STANLEY (1952) has calculated that the carbohydrate-containing lipids strandin (FOLCH, ARSOVE, and MEATH, 1951) and the gangliosides (KLENK, 1941) constitute about 83% of the total cerebrosides determined by this method. This percentage is based on the total grey matter cerebroside values of JOHNSON *et al.* (1949a), which were obtained by analysing unwashed lipid extracts. In the present work the discrepancy may be expected to be smaller, since the extracts were washed once by a procedure which removes all non-lipid contaminants and most of the major contaminating lipid, strandin (FOLCH *et al.*, 1951). Confirmation of the value of the washing procedure was obtained by analysing extracts before and after repeated washing; in one instance the total grey matter cerebrosides fell from 1·22% in the unwashed extract to 0·3% after the third washing, whilst with white matter the corresponding figures were 6·5% to 5·5%. It was not, however, practicable to wash extracts several times as a routine, and all the results reported here were obtained on extracts which had been washed once. To emphasize the less certain nature of figures for cerebrosides in grey matter, the term has been placed in inverted commas when referred to this material.

RESULTS

Relation between the Lipid Content of Preserved Specimens and that of Fresh Specimens

Variation in the lipid content of preserved specimens may be either apparent, resulting from changes in the total weight of the specimens, or real, due to changes in the weight of the lipids themselves. These two possibilities were investigated as follows:

Changes in total weight. Cerebral hemisphere specimens were obtained within 14 hr of death from an adult male of 45 years, whose brain showed no obvious abnormality, and from an idiot child whose brain was macroscopically abnormal. Another human specimen was obtained at a neurosurgical operation within 30 min of removal. All the specimens were immersed in about twenty times their weight of formalin and weighed at intervals up to 24 months. Small specimens of grey and white matter from a rabbit were also studied in this way.

In all of the specimens there was an initial gain in weight, followed by a slow loss (Table 1). The human specimens reached their original fresh weight about 1 year after immersion and subsequently a very slow loss appeared to continue. With the rabbit specimens the initial gain was greater than in the human material, but the ensuing loss occurred at about the same rate.

In the present work all except two of the specimens had been preserved in formalin

VOL 1 1956/

TABLE 1.—CHANGES IN THE WEIGHT OF BRAIN SPECIMENS IMMERSED IN FORMALIN

Source of specimen	Relative weight (original fresh weight = 100) after immersion in formalin for the number of months stated							
	0	½	2	5	12	21	24	45
Normal adult, P.M. 12 g of hemisphere	100	—	112	110	99	—	98	—
Child (2 yr), P.M. 10 g hemisphere: abnormally soft and oedematous	100	—	114	114	100	—	98	—
Adult (neurosurgical operation) 17 g hemisphere	100	127	114	108	101	100	—	—
Rabbit, 1 g cerebral cortex	100	—	143	140	125	113	—	75
Rabbit, 1 g white matter (several areas)	100	—	134	130	125	118	—	80

for periods between 1 and 3 years. It appeared reasonable, therefore, to assume that the weight of the preserved material would be approximately equal to its original fresh weight. This assumption was also adopted with the remaining two specimens which had been in formalin for 11 and 12 years, although in these cases the magnitude of any long-term changes in weight were unknown.

Changes in weight of the lipids. Samples of the specimens used to study weight changes were also analysed for cerebroside content when fresh and after 2 years' immersion in formalin. Two other normal brains, for which data on the fresh lipid content were not available, were analysed for cholesterol and cerebrosides after preservation for 3 and 12 years. Both sets of results are compared with the values for normal fresh brain quoted by JOHNSON *et al.* (1949a) and BRANTE (1949) in Table 2. Figures for the preserved material tend to be somewhat higher than those found in fresh brain by the other workers, particularly in the case of grey matter. This may be due to the more efficient method of extraction used in the present work or the increase may be apparent due to a loss in total weight of the specimens. The latter explanation seems unlikely (except perhaps for the 12 year-old specimen), as in the specimens analysed before and after preservation there was no significant change in cerebroside content. The data, therefore, support the conclusion that the actual content of these two lipids is little affected by formalin preservation for long periods of time. For example, the white matter cerebroside values for the specimen preserved for 12 years were about 20% higher than the published normal values cited in Table 2; if in fact there had been a loss of cerebrosides in this specimen, a decline in its total weight considerably in excess of that suggested by the data in Table 1 would be required to convert this loss into an apparent increase of the magnitude found.

TABLE 2.—CHOLESTEROL AND CEREBROSIDES IN FRESH AND FORMALIN-PRESERVED NORMAL BRAIN SPECIMENS

Results are expressed as g/100 g wet wt. of fresh or formalin-preserved brain. Those quoted for cases N4/49 and 154/40 are the mean of determinations on extracts from the frontal, temporal, and parietal areas of the cerebral hemispheres, followed by the S.D. Values quoted for other authors are followed by the S.E.M.

Source of material or data	Period in formalin (years)	White matter		Grey matter	
		Total cholesterol	Cerebrosides	Total cholesterol	"Cerebrosides"
Previous studies					
JOHNSON *et al.* (1949) (7 cases)	0	4·22 ± 0·17	4·78 ± 0·19	0·99 ± 0·02	0·87 ± 0·24
BRANTE (1949) (12 cases)	0	4·04 ± 0·38	4·68 ± 0·53	0·76 ± 0·10	0·64 ± 0·29
Present studies					
Adult, no obvious abnormality	0	—	5·80	—	0·84
as above	2	3·95	5·50	0·85	0·73
Child (2 years), macroscopically abnormally soft and oedematous	0	—	2·90	—	0·45
as above	2	2·38	2·50	1·03	0·48
Case N4/49	3	3·88 ± 0·31	5·27 ± 0·10	1·19 ± 0·21	1·03 ± 0·19
Case 154/40	12	4·62 ± 0·71	5·72 ± 0·90	1·65 ± 0·20	1·18 ± 0·17

Analysis of Specimens from Abnormal Subjects

Schizophrenia. Samples from the frontal, temporal, and parietal areas of the cerebral hemispheres from three brains were analysed. The results, given in Table 3, are clearly within the normal range, both when compared with values for fresh material and with those for two normal brains preserved for similar periods of time quoted in Table 2. No histological abnormalities were present in these specimens.

Mentally abnormal children. Specimens from the cerebral hemispheres of four subjects were analysed. They were obtained through the co-operation of Dr. L. CROME, and the following brief account of the pathological findings in each case was condensed from his records, which he very kindly allowed me to examine.

*Case M*26/51: the clinical and pathological findings were characteristic of gargoylism. In the grey matter the larger cells were distended and contained faintly sudanophilic granules. There was fibrous gliosis in the white matter. *Case* 39/50: this was a case of megaloencephaly associated with hyaline pan-neuropathy (CROME, 1953). In the grey matter there was a great reduction of nerve cells and gliosis. The white matter showed spongy degeneration and extensive demyelination characterized by an absence of sudanophilic material. Numerous hyaline bodies were present throughout the C.N.S. *Case* 6/51: widespread atrophy of the brain involving particularly the frontal lobes. There was extensive demyelination in the white matter with a large amount of soluble sudanophilic material lying freely and in compound granular corpuscles; widespread fibrous gliosis was present. *Case* 11/51:

the pathological findings in this case were consistent with gargoylism, amaurotic familial idiocy, or neuronal lipoidosis due to some unknown cause. In the grey matter the cortical cells were distorted and ballooned by lipid inclusions, staining faintly pink with Scarlet *R*. There was widespread marginal gliosis. The myelin of the white matter stained poorly throughout the centrum ovale; the individual fibres were beaded, swollen, and pigmented, and there was considerable fibrous gliosis.

TABLE 3.—CHOLESTEROL AND CEREBROSIDES IN BRAIN SPECIMENS FROM THREE CASES OF SCHIZOPHRENIA

Results are expressed as g/100 g wet wt. of formalin-preserved brain, which was taken from the cerebral hemisphere area stated. Figures for ester cholesterol are not included; with the one exception indicated they were all less than 0·01%.

Case	Area	White matter		Grey matter	
		Total cholesterol	Cerebrosides	Total cholesterol	"Cerebrosides"
244/41, age 46 years (11 years in formalin)	Frontal	4·05	5·06	1·22	1·31
	Temporal	3·76	5·35	1·46	1·33
	Parietal	3·68	5·68	1·31	0·96
	Average	3·83	5·36	1·33	1·22
R5/51, age 31 years (1 year in formalin)	Frontal	4·74	4·92	0·93	0·61
	Temporal	4·88	5·18	0·73	0·67
	Parietal	4·78	4·35	0·96	0·68
	Average	4·80	4·82	0·87	0·62
R3/51, age 40 years (1 year in formalin)	Frontal	4·95	5·17	1·10	0·71
	Temporal	4·10	4·55	0·98	0·86
	Parietal	4·54*	5·12	1·14	0·72
	Average	4·53	4·95	1·07	0·76

* Free cholesterol 4·32, ester cholesterol 0·22.

The subjects were all over 2 years of age, when the cholesterol and cerebroside content of brain has normally reached or is very close to adult level (BRANTE, 1949). The results, presented in Table 4, may therefore be reasonably compared to the data for normal material in Table 2. Both lipids were reduced in the white matter of all specimens, but particularly in the sample from case 39/50. Cholesterol esters, normally absent in brain, were increased in the white matter of case 6/51, where sudanophilic material was also present. No significant changes in the lipids in grey matter specimens were observed, even in one case where the histological diagnosis was one of "lipoidosis".

Disseminated sclerosis. Material from two subjects was analysed. Sections of tissue from the areas immediately adjoining those analysed were very kindly prepared

TABLE 4.—CHOLESTEROL AND CEREBROSIDES IN BRAIN SPECIMENS FROM MENTALLY DEFECTIVE CHILDREN

Results are expressed as g/100 g wet wt. of formalin-preserved tissue, which was taken from various parts of the cerebral hemisphere.

Case	Description of specimen	Total cholesterol	Free cholesterol	Ester cholesterol	Cerebroside (white matter) "Cerebrosides" (grey matter)
M26/51, aged 4 years (2 years in formalin)	*Grey matter*: "lipoidosis"	1·12	1·11	0·01	1·02
	White matter: fibrous gliosis	3·00	3·00	0	3·05
39/50,* aged 2 years (3 years in formalin)	*Grey matter*: loss of cells; gliosis	0·75	0·76	0	0·30
	White matter: extreme demyelination; sudanophilic material absent	1·02	1·02	0	0·90
6/51, aged 3 years (3 years in formalin)	*White matter*: demyelination sudanophilic material present;	2·64	1·64	1·00	1·11
11/51, aged 2 years (2 years in formalin)	*Grey matter*: "lipoidosis"	0·99	0·98	0·01	0·69
	White matter: fibrous gliosis; myelin stained poorly	2·94	2·94	0	3·40

* A case of megalencephaly described in detail by CROME (1953).

and examined by Dr. J. B. BRIERLY, who is also responsible for the histological details given below. The extracts were prepared as follows:

Case 27/50. *Sample* 1: a plaque weighing 212 mg was dissected out of a cerebral peduncle. In Herxheimer-stained sections it was seen to be heavily infiltrated with compound granular corpuscles laden with neutral fat. A moderate degree of gliosis was present and there was no residual myelin. *Sample* 2: this consisted of 970 mg of material which included part of the corpus callosum, internal capsule, caudate nucelus, and putamen; the grey matter constituted about 20% of the total specimen. There were small areas of demyelination in the internal capsule tending to occur near the ventricle and showing a heavy gliosis tailing off rapidly into normal white matter; sudanophilic material was absent. The white matter of the corpus callosum was normal. *Sample* 3: 430 mg of sub-cortical white matter from the parietal area was taken. In myelin-stained sections there was a suggestion of pallor of staining and occasional fibres showed some beading, but there was no gliosis or sudanophilic material present. *Case* 79/51. *Samples* 1, 2, *and* 3: two plaques from the mid-brain were dissected out to a depth of 5 mm. One involved about one-third of the right cerebral peduncle and extended into the substantia nigra; the other was smaller and more dorsally situated on the other side; their combined weight was 142 mg (1). For 2, 172 mg of material was taken from an area immediately

adjacent to the smaller plaque in 1; it corresponded in position to that of the larger plaque on the contra-lateral side. The remaining white matter of both peduncles was then dissected out to the same depth and combined to make 3 (149 mg). There was a heavy fibrous gliosis in the plaques and no residual myelin; sudanophilic material, although present, was patchy. In the white matter adjacent to the plaques there was early perivascular gliosis and various degrees of demyelination, seen either as a frank demyelination of groups of fibres or as a diffuse pallor of staining; sudanophilic material was absent. *Samples* 4 *and* 5: 380 mg of a 5 mm wide plaque lining the posterior horn of a lateral ventricle was taken (4). 1·2 g of the adjacent white matter was used for 5. Sudanophilic material and gliosis were patchy in the frankly demyelinated areas, suggesting differing degrees of chronicity of process. There was considerable rarefaction of the myelin around the outer border of the plaque which in some places extended into apparently normal white matter, where early perivascular gliosis was also present.

The analytical results are presented in Table 5. Except in samples 3 and 5 of case 79/51, where surprisingly high values were found, the content of total cholesterol and cerebrosides in the specimens was lower than that found in normal sub-cortical white

TABLE 5.—CHOLESTEROL AND CEREBROSIDES IN BRAIN SPECIMENS FROM TWO CASES OF DISSEMINATED SCLEROSIS

Results are expressed as g/100 g wet wt. of formalin-preserved brain. Demyelinated tissue was located by staining frozen sections by the Weigert Pal method.

Case and No. of extract	Source and histology of specimen	Total cholesterol	Free cholesterol	Ester cholesterol	Cerebrosides
Case 27/50 (2 years in formalin)					
1	Left mid-brain; all demyelinated tissue	3·75	1·36	2·39	2·12
2	Corpus callosum, striatum and internal capsule; demyelinated areas about 20% total	3·47	3·33	0·14	2·89
3	Sub-cortical white matter (parietal)	3·96	3·96	0	3·46
Case 79/51 (1 year in formalin)					
1	Right mid-brain; all demyelinated tissue	3·32	2·38	0·94	3·20
2	Tissue adjacent to above; grossly demyelinated areas absent	3·27	3·23	0·04	3·10
3	Right cerebellar peduncle; grossly demyelinated areas absent	4·92	4·92	0	6·00
4	Periventricular white matter lining left inferior horn. All demyelinated tissue	4·14	2·36	1·78	1·30
5	Tissue adjacent to above; grossly demyelinated areas absent	6·25	6·15	0·10	4·50

matter (Table 2). The cerebrosides were particularly low in two of the plaques (samples 1, case 27/50, and sample 4, case 79/51), but in another plaque which appeared histologically no less chronic (sample 1, case 79/51) they were as high as in the adjacent white matter. A slightly low value for this lipid was also found in the parietal sub-cortical white matter, where no definite histological changes were present. All the specimens containing demyelinated white matter also contained a marked but variable amount of cholesterol esters; these were absent in the apparently well-myelinated areas, even where a fall in the level of cerebrosides was evident.

DISCUSSION

The present results show that the determination of cholesterol and cerebrosides in brain specimens preserved for several years in formalin is a feasible method of investigating the chemical pathology of these lipids. They also indicate, however, that care is needed in interpreting results based on the total wet weight of the preserved specimen, since this changes with time, particularly during the first 6 months of immersion.

In general, the lowest values for both lipids were found in demyelinated white matter, both in the material from children and in that from the two cases of disseminated sclerosis. Smaller reductions were seen in white matter showing mainly fibrous gliosis or where the myelin was only partly attacked. This is consistent with the conclusion of JOHNSON *et al.* (1949a), that cholesterol and cerebrosides are important constituents of the myelin sheath and with the finding that they are reduced in content in peripheral nerve during Wallerian degeneration (JOHNSON *et al.*, 1949b).

The significance of the "cerebroside" figures for grey matter in normal specimens has already been referred to. Here it may be added that in the singly washed extracts of grey matter used in the present work the lipid-bound hexose determined by the method probably consists of about 25% of the strandin, together with the gangliosides and the free cerebrosides derived from the myelin of the sub-cortical nerve fibres. Only in one abnormal specimen, where there was a great reduction in nerve cells (case 39/50, Table 4), was the level of these grey matter "cerebrosides" significantly reduced below normal values.

Cholesterol esters are normally virtually absent from nervous tissue and the small quantity sometimes found is probably derived from blood (SPERRY, 1952). They appear, however, in peripheral nerve during Wallerian degeneration along with neutral fat and other products of myelin breakdown (JOHNSON *et al.*, 1949b). In the present work they were found in all specimens of demyelinated white matter which contained material with the staining properties of neutral fat (sudanophilic material): the severely affected areas from the cases of disseminated sclerosis and the white matter from case 6/51 (Table 4). By contrast they were absent from the demyelinated white matter of case 39/50 (Table 4) where there was also no neutral fat. Similar results to these were reported by CUMINGS (1953), who found cholesterol esters in fresh brain specimens from cases of disseminated sclerosis, and by BLACKWOOD and CUMINGS (1954), who reported their absence in demyelinated white matter lacking neutral fat from a case of diffuse sclerosis of the spongy type.

BLACKWOOD and CUMINGS (1954) interpreted their findings by suggesting that the demyelinated white matter in one of their cases of diffuse sclerosis was the result of an arrest or fault in normal myelination, rather than the active intervention of a demyelinating process. A similar view may be held to apply to case 39/50, since here also

VOL
1
1956/

cholesterol esters and neutral fat, common products of myelin breakdown, were not detected.

SUMMARY

The cerebroside content of two brains was unaffected by immersion in formalin for 2 years. Cholesterol and cerebroside levels in two other brains, which had been preserved in formalin for 3 and 12 years, were found to be close to accepted normal values for fresh brain.

The content of both these lipids was determined in nine abnormal brains, which had been formalin-preserved for periods ranging from 1 to 11 years. No changes were found in specimens from three cases of schizophrenia. In demyelinated specimens from two cases of disseminated sclerosis, low values for total cholesterol and cerebrosides and the appearance of cholesterol esters were found. A similar deficiency in the two lipids, and in one case the appearance of cholesterol esters, were observed in white matter specimens from four cases of mentally abnormal children, whose brains showed a variety of pathological changes.

VOL. 1 56/57

Acknowledgements—I am grateful to Professor H. McILWAIN for his advice and support and to Dr. G. H. SLOANE-STANLEY for helpful suggestions. Some specimens were obtained from the Department of Neuropathology, Maudsley Hospital, through the kind co-operation of Professor A. MEYER and his staff; I am particularly indebted to Dr. J. B. BRIERLY for preparing sections of and reporting on the histology of certain of these. Other specimens, which are gratefully acknowledged, were obtained from Dr. L. CROME, of the Fountain Hospital, S.W.17; Mr. MURRAY FALCONER, Director of the Guys-Maudsley Neurosurgical Unit; and Dr. A. B. BRATTON, Archway Hospital, N.19. I also wish to thank the Board of Governors of the Bethlem Royal and Maudsley Hospital for a grant from their Research Fund.

REFERENCES

BLACKWOOD W. and CUMINGS J. N. (1954) *J. Neuro.* **17,** 33.
BRAND F. C. and SPERRY W. M. (1941) *J. biol. Chem.* **141,** 545.
BRANTE G. (1949) *Acta Physiol. Scand.* **18,** Suppl. 63.
CROME L. (1953) *Brain* **76,** 215.
CUMINGS J. N. (1953) *Brain* **76,** 551.
FOLCH J., ASCOLI I., LEES M., MEATH J. A. and LEBARON F. N. (1951) *J. biol. Chem.* **191,** 833.
FOLCH J., ARSOVE S. and MEATH J. A. (1951) *J. biol. Chem.* **191,** 819.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1948) *Biochem. J.* **43,** 573.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1949a) *Biochem. J.* **44,** 494.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1949b) *Biochem. J.* **45,** 500.
KLENK E. (1941) *Hoppe-Seyl. Z.* **268,** 50.
MILLER B. F. and VAN SLYKE D. D. (1936) *J. biol. Chem.* **114,** 583.
SLOANE-STANLEY G. H. (1952) *Biochem. Soc. Symp.* No. 8, 44.
SPERRY W. M. (1952) *The Biology of Mental Health and Disease* Cassell, London.
SPERRY W. M. and BRAND F. C. (1943) *J. biol. Chem.* **150,** 315.
SPERRY W. M. and WEBB M. (1950) *J. biol. Chem.* **187,** 97.
THANNHAUSER S. J. and SCHMIDT G. (1946) *Physiol. Rev.* **26,** 275.

Journal of Neurochemistry, 1957, Vol. 1, pp. 216-227. Pergamon Press Ltd., London

THE DEVELOPMENT OF THE BLOOD-BRAIN BARRIER*

ABEL LAJTHA
New York State Psychiatric Institute and the Department of Biochemistry; College of Physicians and Surgeons, Columbia University, New York, New York

(*Received* 10 *November* 1956)

SOME years ago, it was found in this laboratory that the intravenous injection of glutamic acid into rats and mice did not lead to an increase of the concentration of the dicarboxylic acid in brain, while the administration of glutamine resulted in a significant rise of the amide concentration in this organ (SCHWERIN *et al.*, 1950; WAELSCH, 1951). This observation impressed upon us the necessity for a more extensive investigation of the properties of the blood-brain barrier to supplement our *in vivo* study of amino acid and protein metabolism in the central nervous system (LAJTHA and FURST, 1955).

Some information on the properties of the blood-brain barrier may be obtained by studying the influence of metabolic inhibitors on the rate of uptake of substances by the brain in the hope that such experiments would lead to an insight into those enzymatic processes, if any, which are responsible for the dynamics of the blood-brain barrier. One may also search for a physiological situation in which the action of the blood-brain barrier is decreased or absent and compare the enzymatic status in such a brain with that in an organ in which the blood-brain barrier is fully operative. Both approaches pressupose that the mechanism responsible for the blood-brain barrier is enzymatic in nature. The immature brain appears to offer a physiological situation in which it may be expected to find the blood-brain barrier absent or not fully effective.

Evidence which may be interpreted as indicating a decreased function of the blood-brain barrier in the immature brain has been summarized and discussed elsewhere (WAELSCH, 1955) and, therefore, only observations of direct bearing on our findings, presented below, will be considered here. It was found (BEHNSEN, 1927) that larger areas of the brain were stained with trypan blue in younger mice than in adult animals, a finding, however, that has been challenged recently (BROMAN, 1949; GRÖNTOFT, 1954). Furthermore, it was found that ferrocyanide was taken up by the brain of the newborn rabbits but not by the brain of newborn guinea-pigs (STERN and PEYROT, 1927). This was interpreted as an indication of the state of maturation of the central nervous system of the respective animal at birth. Of more recent evidence, there may be cited the observation that the rate of entrance into the brain of ammonium hippurate decreases by a factor of 4 in rabbits from the sixth to the twenty-first week after birth (DAVSON, 1955). It has also been shown (FRIES and CHAIKOFF, 1941; BAKAY, 1953) that the rate of entrance of phosphate (P^{32}) decreases during

* Supported in part by grants from the Supreme Council, 33° Scottish Rite Masons of the Northern Jurisdiction, United States of America, and from the National Institute of Neurological Disease and Blindness (Grants B-226-C and B-557), National Institutes of Health, United States Public Health Service.
A preliminary report on some of the findings has appeared (WAELSCH, 1955).

maturation. Although these experiments may support the thesis that the blood-brain barrier is absent or functioning to a lesser degree in the immature brain, it is important to remember that phosphate is to the largest part a component of the intracellular compartment and that it is an actively metabolized tissue component. It could be visualized that its increased uptake by the immature animal is a reflection of the metabolic situation of the organ and not primarily a measure of the function of the blood-brain barrier.

We wish to report on a comparative study of the rate of uptake of chloride-36 and thiocyanate by brain, muscle, and liver of chick embryos and chicks of different ages after hatching as well as of newborn and older rats. In the course of this study, data on the chloride space and water content of the organs of these animals were collected. Chloride and thiocyanate were selected, since these ions are metabolically inert and are assumed to be, in organs other than the brain, limited to the extracellular fluid phase.

EXPERIMENTAL

Animal experiments

Chicks of the single-combed white Leghorn strain and rats of the Sprague-Dawley strain were used. Adult chickens were injected intravenously in the wing vein, young chicks up to about 4 weeks of age and rats were injected intraperitoneally. For the intravenous injection of chick embryos, the top of the egg over the air chamber was sawn off, and the allantoic vessels injected with a fine needle or, in younger embryos, with a finely drawn capillary. Care was taken to withdraw the needle slowly in order to avoid bleeding. The egg cap was replaced with scotch tape to prevent excessive dehydration. Adult animals were kept at room temperature for the experimental periods, young animals in a 35°C warm room and the eggs in a 37°C incubator.

Collection of blood

Blood was obtained from chickens after decapitation and from the embryos by puncture of the chorio-allantoic vessels. The blood from rats was obtained after decapitation. After clotting, the blood was centrifuged and the serum pipetted off.

Chloride determination

The organs were homogenized in 0·75 N-HNO_3 (10 volumes) and the homogenate kept at room temperature for 5 min and centrifuged. The precipitate was homogenized again in 5 volumes of 0·6 N-HNO_3, centrifuged, and the procedure repeated twice more. The combined extracts were once more centrifuged at high speed (8000 × g) and concentrated nitric acid was added to the supernatant solution to a final concentration of 2 N. The solution was cleared by heating it for 30 min in a boiling-water bath. Chloride was precipitated from this hot solution by the addition of a 0·5 N silver nitrate solution. The precipitate was washed with small portions of water, ethanol, and ether, dried at 105°, and weighed.

In view of a recent report (SHENK, 1954) that water or buffer solution does not extract all of the tissue chloride, the results of the above method were compared in a separate set of experiments with values obtained by the Carius method. An aliquot of the tissue was extracted with cold nitric acid, as described above, and another was oxidized in the Carius bomb tube according to SHENK's method. No significant differences were found between the results of the two methods when applied to blood, blood plasma, brain, liver, and muscle.

For the determination of chloride in the whole chick embryo, the eggs were opened, the embryo was quickly separated from the rest of the egg, exsanguinated, dried with tissue paper, and weighed. It was then homogenized in cold nitric acid, as described above.

Determination of water content

The organs were homogenized in 5 volumes of 95% ethanol and the homogenate dried to constant weight at 115°C.

Measurement of radioactivity

In those experiments in which chloride-36 was administered to animals, the silver chloride was prepared as described above. The activity of the sample after plating on glass pans was determined in a gas flow counter (Tracerlab) and corrected for self-absorption.

Determination of thiocyanate

For the extraction of thiocyanate, the tissues were homogenized in 20 volumes 95% alcohol and re-extracted twice more with 95% alcohol. Thiocyanate was determined in acid medium with the $FeCl_3$ reaction and the colour density read at 520 mμ (GOLDSTEIN, 1950).

RESULTS

The chloride concentration of the whole chick embryo diminished during the period under study, i.e. from the sixth day of incubation to the day before hatching it decreased to less than one-half and was, at the end of incubation, only slightly above the average chloride concentration found for the whole fertilized egg (Table 1). From the eighth to fifteenth day of incubation when the chloride concentration of the embryos dropped from approximately 87 to 48 meq/kg, the blood and its serum showed a constant chloride concentration which was within the range found in adult chicken (Tables 1 and 2).

TABLE 1.—CHLORIDE CONTENT OF CHICK EMBRYOS AND THEIR BLOOD

Incubation days	Stage*	Chloride meq/kg	
		Embryo	Blood
6	25	107	
6	26	94	
7	27	98	
7	28	108	
7	29	87	
8	30	88	
8	31	87	116
8	32	83	
9	34	77; 62	125
10	35	75	108; 85
11	36		89; 93
13	37	70; 69	110; 116
13	38	76	
14	40	51; 56	
15	41	48	118; 97; 113
16	42	42; 40	122; 108
20	45	45; 36	
Eggs fertilized		33; 35; 32; 33	
After hatching days			
50			107; 108
360			91; 89

* Stages according to HAMBURGER and HAMILTON (1951).

The decrease of the chloride concentration and water content of the whole embryo during development was reflected in parallel changes in brain, liver, and muscle, a finding which also applies to the organs of rats from birth to adulthood (Table 2).

TABLE 2.—CHLORIDE AND WATER CONTENT OF ORGANS OF CHICKS AND RATS DURING DEVELOPMENT

Days	Serum chloride meq/kg	Brain Chloride meq/kg	Brain Water %	Liver Chloride meq/kg	Liver Water %	Muscle Chloride meq/kg	Muscle Water %
Incubation			*Chick embryo*				
10			91		81		
13			90		79		
14	119	48	89	47	75	60	89
18	110	37		34		38	
20	121	39	86	34	74	36	83
After hatching			*Chick*				
1	104	33		26		30	
3	108	31	84	26	74	29	
5			84		72		
7	121	34	82	26	67	30	80
12			81		70		
26	111	28		23		22	
42	122	30		24		21	
245			78		67		77
			Rat				
2	108	44		43		64	
5	118	43		39		45	
6			87		74		82
10	120	42		36		37	
20	133	43		33		30	
30	115	35		26		22	
70	113	32		25		20	
210			82		69		
360	109	30	78	24	69		77

All figures rounded off to the next whole number. Each determination average of duplicates.

The specific activity of the chloride of blood, brain, and other organs of chick embryos, chicks after hatching, and rats of different ages after the injection of $NaCl^{36}$ is shown in Table 3. In the experiments with chicks, the specific activity of the blood chlorides is given (column 5) and the specific activity of chlorides of brain, liver, and muscle is expressed as percentage of this value (columns 6–9). In the experiments with rats the specific activity of the liver chlorides was taken as 100. It can be seen that with increasing age of chick embryos, chicks, or rats, the time-interval for the attainment of equilibrium between blood chlorides and brain chlorides was increasingly longer, while it did not change for liver or muscle chloride.

Similar results were obtained after the injection of thiocyanate into chick embryos, chicks after hatching, and rats of different ages (Table 4). In columns 8 and 9 the thiocyanate concentrations (in μmole/g) found in the blood, brain, and liver are expressed as percentage of the chloride concentrations (μmole/g) of these organs. The chloride values are taken from Table 2 and the percentages are calculated without

TABLE 3.—RATE OF PENETRATION OF CHLORIDE (Cl^{36}) INTO THE ORGANS OF CHICK EMBRYOS, CHICKS, AND RATS

Animals	Age in days	Average weight of animal (g)	Duration of experiment (hours)	Blood counts/ min/mg AgCl	Specific activity of Cl Blood chloride = 100				Ratio liver/ brain	Way of injection
					Blood	Brain	Liver	Muscle		
1	2	3	4	5	6	7	8	9	10	11
Chick embryo	13	4·97	1	65·2	100	92	94	93	1·02	IV
Chick embryo	15	11·4	1	62·1	100	100	110		1·1	IV
Chick embryo	15	11·9	1	53·9	100	81	89	101	1·1	IV
Chick embryo	17	15·9	1	59·2	100	57	99	97	1·7	IV
Chick embryo	17	14·8	1	45·4	100	45	88	101	2·0	IV
Chick embryo	20	26·2	1	60·1	100	50	91	100	1·8	IV
Chick	2	27·4	1	26·1	100	46	92		2·0	IM
Chick	28	268	1	54·0	100	33	89		2·7	IV
Chick	28	296	3	55·5	100	95	96		1·0	IV
Chick	56	524	1	49·0	100	31	94		3·0	IV
Chick	56	552	4	42·0	100	76	97		1·3	IV
Chick	210	2230	6	40·0	100	95	91		1·0	IV
Rat	9	13·5	0·5	38·7		104	100		1·0	IP
Rat	60	128	0·5	31·1		51	100		2·0	IP
Rat	60	137	1	41·2		118	100		0·85	IP

Each value is the average of two animals. 0·8% NaCl solution was used (1 μc/c.c.), 0·001 c.c. corresponding to 0·001 μc, and 8 γ NaCl was injected per gram body-weight, an amount which corresponds to about 2% of serum chloride and serum volume. Method of injection IV = intravenous; IP = intraperitoneal; IM = intramuscular.

VOL 1 1956/

TABLE 4.—UPTAKE OF THIOCYANATE BY BRAIN AND MUSCLE OF CHICK EMBRYOS, CHICKS, AND RATS

Days	Duration of experiment (min)	NaCNS injected (μmole/g)	CNS^- in tissue (g)			$\frac{100 \times CNS^-}{(Cl^-)}$			
			Blood (μmole)	Brain (μmole)	Liver (μmole)	Blood	Brain	Liver	Liver / Brain
Incubation				*Chick embryos*					
14	15	2·5	6·0	2·3	2·3	7·5	4·8	4·9	1·0
15	15	2·5	7·3	2·6	3·1	9·1	5·8	7·0	1·2
18	15	2·5	6·9	2·7	2·6	8·6	7·3	7·7	1·1
20	15	2·5	6·5	1·3	1·4	8·1	3·3	4·1	1·2
20	60	2·5	5·5	1·7	1·5	6·8	4·4	4·4	1·0
After hatching				*Chicks*					
1	15	5·0	11·3	2·0	3·4	14·0	6·1	13·0	2·1
2	60	5·0	14·4	1·9	3·1	18·0	6·0	12·0	2·0
2	120	5·0	12·4	4·4	4·0	15·4	14·0	15·0	1·1
56	60	5·0	11·4	1·7	2·7	14·2	5·7	11·0	1·9
56	120	5·0	10·3	2·4	2·5	12·8	8·0	10·0	1·3
Days				*Rats*					
6	60	3·0	7·9	3·4	2·6	12·5	7·9	6·8	0·9
27	60	3·0	5·6	0·8	1·0	8·8	2·3	3·8	1·7
45	60	3·0	8·4	1·1	1·9	13·3	3·1	7·3	2·4
365	60	3·0	5·2	0·6	1·4	8·2	2·0	5·8	2·9

Hematocrit of chicken blood assumed to be = 30% and of rats = 45%. Chloride concentration in chicken serum 115 meq/kg and in rat serum 117 meq/kg. For the calculation it is assumed that all thiocyanate is present in the blood serum.

Each value is the average of duplicate determinations from three separate experiments carried out on two chick embryos each or single chicks or rats.

All experiments were carried out with intravenous injection except with the 1- and 2-day chicks, which were injected intramuscularly.

consideration of a displacement of chloride by thiocyanate. In the last column the ratios of the percentages as calculated for liver and brain are given (column 9/column 8). These ratios differ very slightly from those obtained directly from the thiocyanate concentrations in brain and liver, since the chloride concentrations in both organs are close. The increase of these ratios with age demonstrates that less and less of the chloride space of the brain is penetrated by thiocyanate.

Whereas only minimal amounts of chloride were administered and variations of this amount did not play a role in the rate of uptake of chloride by the tissues, it can be seen from Table 5 that with larger amounts of thiocyanate the thiocyanate concentration in brain relative to that in the liver became increasingly higher in animals of the same age and under comparable experimental conditions. The values of the last four columns have been arrived at by the same calculations as used for the values in the corresponding columns of Table 4. A doubling of the dose of thiocyanate led to a considerably larger uptake relative to that by the liver. Since the uptake of thiocyanate by the liver roughly parallels the dose administered, it is unlikely that

TABLE 5.—DEPENDENCE OF THIOCYANATE UPTAKE BY BRAIN AND LIVER (CHICK) ON ITS BLOOD LEVEL

NaCNS administered per animal (μmole)	Average weight of animal	NaCNS (μM/g)	CNS^- in tissue (g)			$\frac{100 \times (CNS^-)}{(Cl^-)}$			
			Blood (μmole)	Brain (μmole)	Liver (μmole)	Blood	Brain	Liver	$\frac{\text{Liver}}{\text{Brain}}$
20	33	0·6	1·3 1·7	0·02 0·09	0·27 0·31	1·6 2·1	0·07 0·3	1·0 1·2	14 4
40	33	1·2	3·7 3·2	0·23 0·16	1·04 0·71	4·6 4·0	0·7 0·5	4·0 2·7	5·7 5·4
100	32	3·1	10·2 8·0	1·07 1·40	2·37 2·02	12·6 10·0	3·4 3·4	9·1 7·8	2·7 2·3
200	31	6·5	19·8 16·1	3·63 2·72	5·28 3·57	24·6 20·0	11·7 8·8	20·3 13·7	1·7 1·6
300	30	10·0	24·4	6·77	7·45	30·3	21·8	28·8	1·3

For each of the nine experiments the organs of two 3-day-old chickens were pooled and the determination carried out in duplicate. The animals were killed 40 min after intramuscular injection of the thiocyanate solution.

approaching saturation by the liver resulted in an apparent relative higher uptake by the brain. In the experiments with chick embryos and chicks after hatching, the latter group received twice the amount of thiocyanate per gram (Table 4). Therefore, the restriction of uptake of thiocyanate by brain with development may actually be greater than found in the experiments.

DISCUSSION

Our findings with representatives of two classes of vertebrates show that with increasing age the chloride space of the brain is penetrated at an increasingly slower rate by chloride and thiocyanate supplied from the circulating blood. In order to obtain a clearer picture of these findings, it appears desirable to consider first the changes in chloride and water content of the developing organs.

With a constant chloride concentration in blood or blood serum, the chloride concentration of brain and liver of the chick decreases by about 30% and 45% respectively from the fourteenth day of incubation to the first day after hatching and remains nearly constant for the next 40 days, which was the period of observation. During the period of greatest drop in chloride concentration, the water content of both organs decreases by less than 10%. The decrease in chloride concentration of the leg muscles from the fourteenth day of incubation until the seventh day after hatching is considerably greater than found in liver and brain and continues beyond the time when the chloride concentration in the two latter organs has already become stabilized. Again, the decrease in water content is only a fraction of the decrease in chloride content of the muscle. It is interesting to note that the drop in chloride concentration in chicken breast-muscle occurs at a considerably later period, i.e.

VOL 1 1956/

after the third day after hatching (BARLOW *et al.*, 1947). The uneven shrinkage of chloride and water content of organs during development has been interpreted as a decrease in extracellular, with simultaneous increase in intracellular, fluid space.

Assuming for the moment that all chloride is in the extracellular compartment, our data may be used for a calculation of the extracellular fluid phase of brain, muscle, and liver in the developing chick and rat (Fig. 1). The following values are found for these organs of the adult chicken and rat respectively: Brain, 23% and 26%; liver, 19% and 20%; muscle, 16% and 16%. Experiments on the distribution of iodide, thiocyanate, bromide, and chloride in the central nervous system and the spinal fluid (WALLACE and BRODIE, 1939; MANERY, 1954) suggest that the interstitial fluid of the brain tissue has the same relationship to spinal fluid as the blood plasma to the extracellular fluid phase of other tissues. If the calculation of the extent of extracellular compartment would be based on the chloride content of the spinal fluid of large mammals, an extracellular chloride space of 20% and 22% respectively would be obtained.

The question to what extent chloride space corresponds to extracellular fluid phase is still under discussion (cf. MANERY, 1954; DEAN *et al.*, 1952). It appears that the largest part of intracellular chloride is to be found in blood cells, gastric mucosa, and lung tissue. Sucrose space of the body is smaller than chloride space, and this difference may be due, to a large part, to the inability of sucrose to enter the cerebrospinal fluid and the gastrointestinal juices (AMBERSON *et al.*, 1938; MANERY and HASTINGS, 1939; NICHOLS *et al.*, 1952; WALSER *et al.*, 1953; GAMBLE *et al.*, 1953). It was shown several years ago and recently confirmed, that in muscle free of connective tissue the chloride space agrees closely with the histologically determined extracellular space (BARLOW and MANERY, 1954), a finding made previously for liver (TRUAX, 1939). On the basis of results obtained with different techniques, there appears to be general agreement that the chloride space in liver and muscle corresponds closely to the extracellular fluid phase. It has been shown recently that the high chloride content of breast muscle of the three- to four-day-old chick corresponds to a large extracellular compartment which during maturation is displaced by cells as the muscle develops (BARLOW and MANERY, 1954).

Brain presents a considerably more complex situation than muscle or liver, since in this organ the blood-brain barrier is supposed to function between the extracellular compartment and the circulating blood. Whereas in some investigations correspondence of chloride and extracellular fluid phase has been assumed tacitly (FLEXNER and FLEXNER, 1949), in others, results were interpreted as indicating a large portion of intracellular chloride (AMBERSON *et al.*, 1938). This conclusion was based on the inability of sulphate to replace the chloride of the brain, but was drawn without any consideration of the existence of the blood-brain barrier. Recently (ALLEN, 1955), it has been claimed that the extracellular space of brain tissue slices is about 14·5% and 17% respectively, on the basis of the uptake of inulin or ferrocyanide by these slices. A value of 15% would correspond to an intracellular chloride content of 42% of the total chloride of the organ. It appears difficult to define a state of metabolism with brain slices which would simulate truly the *in vivo* situation with its maintenance of different compartments. These observations throw some doubt on the size of the extracellular fluid phase of the brain.

At present it is impossible to state whether the extracellular compartment of the

brain corresponds in its properties to those of liver and muscle or whether it is represented to a larger or smaller degree by elements such as glia, ground substance, myelin, etc. Even if it is assumed that most of the chloride is excluded from the neurons, it cannot be assumed that in the adult brain the chloride space corresponds to the extracellular fluid phase. It is with these restrictions in mind that in this communication the term chloride space is used.

The observations on decrease in chloride content and consequently in chloride space of the organs of the two animals studied during development supplement the data of YANNET and DARROW (1940) on young and mature cats and of FLEXNER and FLEXNER (1949, 1950), who demonstrated the shrinkage of chloride space and increase in intracellular fluid phase in the brain cortex and liver of guinea-pig during embryonic development. It may be noted that in our study the extracellular chloride

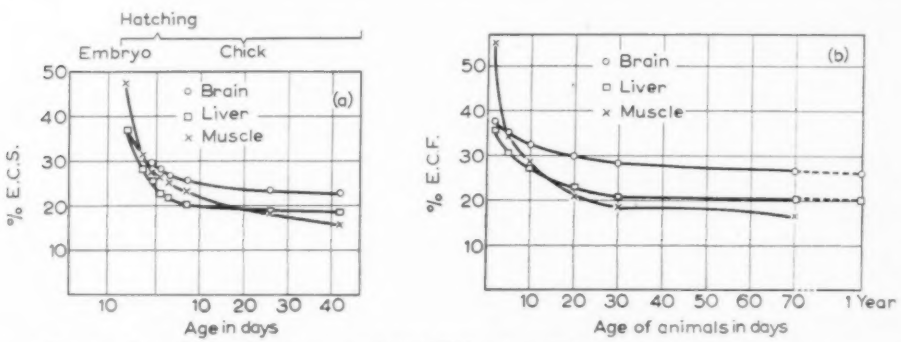


FIG. 1.—Extracellular chloride space of chick (IA) and rat (IB) organs during development.

$$\text{Extracellular chloride space (ECF)} = \frac{\text{Cl in tissue meq/kg} \times 0{\cdot}977 \times 0{\cdot}94}{\text{Cl meq/kg in serum}}$$

(94% serum water, 0·977 distribution ratio for chloride (cf. BARLOW and MANERY, 1954)).

space does not go through a maximum as found for the brain cortex of guinea-pigs around the fortieth day of gestation (FLEXNER and FLEXNER, 1949, Fig. 1). This difference may be explained either by the different time schedule used in our experiments or by the fact that we analysed the whole brain, while the FLEXNERS were concerned with the brain cortex only. It should be noted that our analyses were carried out on the organs without the removal of fat. Whereas the lipid content of the liver does not change significantly during the period under study, the lipid content of the brain increases considerably owing to the myelinating process which starts in the rat brain around the tenth day after birth. In the adult brain the lipids represent an essential and constant part of the tissue structure (cf. LOWRY *et al.*, 1946). In the rat brain the chloride space decreases from the second day to the tenth day after birth by about the same percentage as it does between the tenth and thirtieth day, during which period the fatty acid content of this organ doubles owing to myelination (WAELSCH *et al.*, 1941).

For the purpose of visualization of the changes in development, the "extracellular compartment" (Fig. 1) was calculated under the assumption that it corresponds to the chloride space, i.e. that all chloride is extracellular. With a portion of chloride being intracellular, the relative decrease of the extracellular space during development

would be the same only if the ratio of the chloride concentration in the two compartments would be constant during the periods under study.

The declining rate of chloride-36 uptake by the brain when compared with the uptake by liver and muscle proceeds without any noticeable break. It appears that in the chick shortly after hatching but at a later date than when the near-constancy of the chloride space is established, the rate of uptake becomes fixed. The values reported are somewhat in error, since no account was taken of the amount of blood remaining in the organs of the decapitated animals. Since the difference between the specific activity of blood and organ chloride was not large, a considerable admixture of blood would not seriously change the values. It would express itself mainly in the adult animals in which the percentage penetration may appear slightly higher than it actually is. A more serious question for the interpretation of the data arises from the possibility that an increasingly larger portion of the chloride of the brain resides in the cellular elements with increasing age (AMBERSON, 1939). In this connection, the claim may be recalled that a portion of brain potassium becomes non-diffusible with maturation of the organ (KATZMAN and LEIDERMAN, 1953). It is apparent that much less than 15% of the brain would have to be extracellular chloride space in order to explain a specific activity of only one-third of that of the blood or liver in an experiment of 1 hour duration. The value of approximately 30% penetration of the chloride space of the adult brain by isotopic chloride agrees well with the results obtained with rabbits (MANERY and HAEGE, 1941). In these experiments complete penetration of the chloride space of the liver by isotopic chloride was obtained within 11 min, while only 5% of the chloride space of the brain was penetrated during the same period. If this value is taken as a measure of the chloride of the brain which will exchange at a rate comparable with that of liver chloride, 95% of the brain chloride would have to be assumed to be intracellular. Assuming any larger chloride space would lead to the postulation of the development of a barrier between blood and brain, or alternately to the assumption that the chloride space of the immature brain is occupied during development with material into which the uptake of chloride is delayed. With comparable distribution of chloride between intra- and extracellular compartment in the immature as in the mature brain, it follows that a barrier would be absent or function less efficiently in the immature brain.

Although essentially the same reasoning applies to the thiocyanate experiments, it should be recalled that there is considerable evidence that the thiocyanate space is larger than the chloride and sodium space (WINKLER *et al.*, 1943). An indication that thiocyanate may not be restricted to the chloride space may be seen also in the fact that increasing doses lead to increased rate of entrance of the anion into the brain relative to that into the liver. An alternate explanation may be that at higher levels thiocyanate affects the blood-brain barrier in a way which leads to a suppression of its efficiency. It is of some interest in this connection that thiocyanate administered to humans has been observed to produce toxic delirium once the blood-level surpasses 14 mg% (for a recent summary see DOMZALSKI *et al.*, 1953). The possibilities may be considered that the toxic effect may be due to an action of thiocyanate or one of its derivatives on the metabolism of the brain or on the blood-brain barrier, thereby upsetting the supply to and release of metabolites from the brain.

While the amounts of chloride (Cl^{36}) administered were small enough to permit

the assumption that no net uptake but only exchange of chloride was measured, the uptake of the larger amounts of thiocyanate given can occur with or without corresponding displacement of chloride.

Finally, the question may be raised to what degree changes in blood-flow occurring during development may affect the change in rate of penetration of chloride and thiocyanate into the brain. Since, as found in our experiments and also by previous investigators, chloride and thiocyanate migrate only slowly from the blood-stream into the adult brain, and in addition, the concentration of chloride in blood serum is about three times that found in the brain, it is probable that changes in blood-flow will not affect seriously the rate of exchange of chloride between blood and brain.

The findings presented in this paper based on the behaviour of metabolically inert anions assumed to be components of the extracellular phase in organs other than the brain, appear to offer strong evidence that their rate of uptake by the brain is slowed down during development. The mechanism underlying this change in rate of uptake is obscure. While it may be due to the development of a blood-brain barrier, changes in the composition of the extracellular chloride space of the central nervous system may also represent a major contributing factor.

VOL
1
1956/

SUMMARY

The uptake of chloride-36 and thiocyanate by brain, liver, and muscle of chick embryos, chicks after hatching, and of rats at different ages after birth has been investigated. Relative to the uptake by liver and muscle, the rate of uptake by the brain of the two anions decreases with increasing age of the animal. The findings are discussed in relation to the concepts of the blood-brain barrier and the chloride space of the brain.

Acknowledgement—It is a pleasure to thank Professor HEINRICH WAELSCH for his continued interest and advice in these investigations and for his helpful criticism in the preparation of the manuscript.

REFERENCES

ALLEN J. N. (1955) *Arch. Neurol. Psychiat.* **73,** 241.
AMBERSON W. R., NASH T. P., MULDER A. G., and BINNS D. (1938) *Amer. J. Physiol.* **122,** 335.
BAKAY L. (1953) *Arch. Neurol. Psychiat.* **70,** 30.
BARLOW J. S., SLINGER S. J. and MANERY J. F. (1947) *J. Gerontol.* **2,** 110.
BARLOW J. S. and MANERY J. F. (1954) *J. Cell. and Comp. Physiol.* **43,** 165.
BEHNSEN G. (1927) *Z. Zellforsch.* **4,** 515.
BROMAN J. (1949) *The Permeability of the Cerebrospinal Vessels in Normal and Pathological Conditions* E. Munksgaard, Copenhagen.
DAVSON H. (1955) *J. Physiol.* **129,** 111.
DOMZALSKI C. A., JR. KOLB L. C., and HINES E. A., JR. (1953) *Proc. Staff Meet. Mayo Clinic* **28,** 272.
FELLERS F. X., BARNETT H. L., HARE K., and MCNAMARA H. (1949) *Pediatrics* **3,** 622.
FLEXNER L. B. and FLEXNER J. B. (1949) *J. Cell. and Comp. Physiol.* **34,** 115.
FLEXNER L. B. and FLEXNER J. B. (1950) *Anatom. Record* **106,** 413.
FRIES B. A. and CHAIKOFF I. L. (1941) *J. biol. Chem.* **141,** 478.
GAMBLE J. L. JR., ROBERTSON J. S., HANNIGAN C. A., FOSTER C. G., and FARR L. E. (1953) *J. clin. Invest.* **32,** 483.
GOLDSTEIN F. (1950) *J. biol. Chem.* **187,** 523.
GRÖNTOFT O. (1954) *Acta. pathol. microbiol. scand. Suppl.* **100,** 47.
HAMBURGER V. and HAMILTON H. L. (1951) *J. Morphol.* **88,** 49.

KATZMAN R. and LEIDERMAN P. H. (1953) *Amer. J. Physiol.* **175,** 263.
LAJTHA A. and FURST S. (1955) *Symp. Neurometabolism* (in press).
LOWRY O. H., HASTINGS A. B., MCCAY C. M., and BROWN A. N. (1946) *J. Gerontol.* **1,** 345.
MANERY J. F. (1954) *Physiol. Rev.* **34,** 334.
MANERY J. F. and HASTINGS A. B. (1939) *J. biol. Chem.* **127,** 657.
MANERY J. F. and HAEGE L. F. (1941) *Amer. J. Physiol.* **134,** 83.
NICHOLS G. JR., NICHOLS N., WEIL W. B., and WALLACE W. M. (1952) *J. clin. Invest.* **31,** 652.
SCHWERIN P., BESSMAN S. P., and WAELSCH H. (1950) *J. biol. Chem.* **184,** 37.
SHENK W. D. (1954) *Arch. Biochem. Biophys.* **49,** 138.
STERN L. and PEYROT P. (1927) *C. Rend. Soc. Biol.* **96,** 1124.
TRUAX F. L. (1939) *Amer. J. Physiol.* **126,** 402.
WAELSCH H. (1951) *Adv. Protein Chemistry* **6,** 299.
WAELSCH H. (1955) in *Biochemistry of the Developing Nervous System* (Ed. H. WAELSCH) p. 187. Academic Press, New York.
WAELSCH H., SPERRY W. M., and STOYANOFF V. A. (1941) *J. biol. Chem.* **140,** 885.
WALLACE J. B. and BRODIE B. B. (1939) *J. Pharmacol. Exp. Therap.* **65,** 220.
WALSER M., SELDIN D. W., and GROLLMAN A. (1953) *J. clin. Invest.* **32,** 299.
WINKLER A. W., ELKINTON J. R., and EISEMAN A. J. (1943) *Amer. J. Physiol.* **139,** 239.
YANNET H. and DARROW D. C. (1940) *J. biol. Chem.* **134,** 721.

OL.
1
6/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 228-233, Pergamon Press Ltd., London

THE FREE HISTIDINE, HISTAMINE, AND ARGININE CONTENT OF THE RAT BRAIN

DORIS H. CLOUET,* M. K. GAITONDE,* and D. RICHTER
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff, Wales

(*Received* 4 *October* 1956)

VOL
1
1956/

THE free amino acid level in the rat brain is second only to that in the kidney, which is the highest in the body (FRIEDBERG and GREENBERG, 1947). The significance of the high amino acid content of the brain is not yet clear, but experiments with isotopically labelled amino acids have shown that free amino acids are rapidly incorporated into the brain proteins (GAITONDE and RICHTER, 1955, 1956). Two amino acids are present in particularly high concentration in the brain: glutamic acid, which appears to serve a special role, since its level varies with changes in the physiological state (ANSELL and RICHTER, 1954; DAWSON, 1953; VRBA, 1956); and aspartic acid, which is present mainly as the acetyl derivative in rat brain (TALLAN, 1956). The basic amino acids, arginine and histidine, are also of interest in view of the work of WILLIAMS, SCHURR, and ELVEHJEM (1950), who reported marked changes in the concentrations of these amino acids in the brain of animals subjected to cold stress and to physical exercise.

Although chemical methods are available for estimating arginine and histidine, their application to tissue extracts is difficult, owing to the low concentrations of these compounds normally present in the tissue and the lack of specificity of the methods. In the present investigation the specificity of the methods was improved by the preliminary chromatographic separation of the basic amino acids on a resin column: this enabled histidine to be separated from histamine and arginine from glycocyamine before their determination in the same tissue sample. Using this method, a study was made of the normal levels of histidine, arginine, and histamine in the rat brain; the effects of anaesthesia, hypoglycaemia, excercise, and cold stress were examined.

METHODS

Male Wistar albino rats of about 190 g were divided into six groups of six rats each: series (a) control and hypoglycaemic, series (b) control, hypoglycaemic, exercised, and chilled. Series (b) differed from series (a) in that the rats in series (b) were anaesthetized before killing. Anaesthesia was produced by the subcutaneous injection of 10 mg of sodium amytal/100 g of body weight. Hypoglycaemia was produced by the intraperitoneal injection of insulin into the rats in 4-unit doses until coma was obtained. The body temperature of the anaesthetized rats, and those in coma, was maintained. The exercised rats were kept for 6 hours in a drum rotating so that the average distance travelled by each rat was 12·5 m/min. The chilled animals were kept for 6 hours in a refrigerator at 2°C. The rats were fasted for 12 hours before they were killed.

After the appropriate treatment, the rats were dropped quickly into a large beaker of liquid oxygen. The brains were removed, pulverized while still in the frozen state in a cooled metal crusher, and stirred into 10% (w/v) trichloroacetic acid. An ether-extracted acid-soluble filtrate was prepared.

Chromatographic separation. Glass columns (1·6 cm internal diameter × 12 cm long) were filled to a depth of 4 cm with Zeo-Karb 225 (Permutit Co.), a sulphonated polystyrene resin. The resin was used in the H^+ form and regenerated by the passage of 1 N-NaOH followed by 2 N-HCl until an acid eluate

* Present address: Institute of Living, 200 Retreat Avenue, Hartford, Connecticut, U.S.A.

was obtained; it was then washed with water until the effluent was neutral. It was convenient to operate eight columns simultaneously.

The sample to be analysed (in this case an ether-extracted trichloroacetic acid filtrate) was adjusted to pH 5–7, with 0·1 N-NaOH, made up to a 10 ml volume with water, and transferred to the column. Fractions of the eluates were collected in successive 10 ml aliquots at a rate of elution of 35 ml/hr. Citrate buffer (80 ml of 0·5 M, pH 5·4) was run through the columns, followed by 40 ml of 1 N-NaOH. The resulting twelve tubes of eluate were numbered consecutively.

With this procedure, the acidic and neutral amino acids and histidine were eluted by the citrate buffer, while arginine and histamine remained on the column until the elution with NaOH. Histidine was found in tube number 5, glycocyamine in tubes 4 to 6, histamine in tubes 9 and 10, and arginine in tubes 10 and 11. Tyrosine, which reacts with the Pauly reagents under our conditions at one-tenth of the molecular colour of histidine, was found in tube number 3; it was therefore readily separated from histidine.

The separation of glycocyamine and arginine as described above was unsatisfactory for the quantitative determination of glycocyamine because high blank values were found in the colorimetric analysis of the citrate eluates. Therefore, in an additional group of six rats, glycocyamine was separated from arginine in one stage by using Zeo-Karb 225 in the sodium form: by this method arginine was adsorbed on the resin and glycocyamine was quantitatively recovered in the effluent. This method was suitable for micro quantities of the material (5–20 μg). To provide a comparison kidney extracts from the same rats were also examined.

Determination of histidine and histamine. The Pauly reaction with diazotized sulphanilic acid is usually performed in sodium carbonate solution, as described by MACPHERSON (1942). It was found that the colour reaction became more reproducible and more sensitive if (1) the pH of the sample was controlled, and (2) the time of colour development was kept short.

Procedure. A 4 ml sample of the 10 ml eluate in a 10 ml graduated tube was brought to pH 6·8 with N-HCl or N-NaOH, using B. D. H. "narrow range" indicator paper. The solution was shaken with 1 ml of 1% (w/v) sulphanilic acid in N-HCl and 1 ml of 5% (w/v) sodium nitrite. After 20 min at room temperature, 2 ml saturated sodium carbonate solution were added. The mixture was shaken carefully to minimize the formation of bubbles of CO_2 and the volume was quickly made up to 10 ml. The colour was read at 480 mμ within 30 sec of adding the sodium carbonate.

In the case of highly buffered samples, such as the citrate buffer eluates, 0·5 ml 6 N-HCl was added before the addition of the sulphanilic acid, and this was neutralized by the addition of 0·5 ml 6 N-NaOH immediately before the addition of the sodium carbonate solution.

The range of histidine and histamine determined by this method was from 0·5–20 μg.

Determination of arginine and glycocyamine. DUBNOFF's modification (1941) of the Sakaguchi reaction was slightly modified in the procedure described below, which covers a range of 2–30 μg of arginine or glycocyamine. Higher quantities were determined by using correspondingly higher amounts of the naphthol-urea mixture and of the hypobromite solution. The method was reproducible when the hypobromite solution was delivered *rapidly under pressure and followed at once by shaking.*

Procedure. A 3 ml sample was neutralized (to pH 7·0–7·5), using a wide-range indicator paper. The volume was brought to 4 ml with distilled water and 1 ml of 10% (w/v) KOH was added. After mixing, the tube was placed in an ice-bath for 10 min. The following reagents were added, mixing the contents after each addition: 0·2 ml of naphthol-urea mixture containing equal volumes of 0·2% (w/v) α-naphthol in absolute ethanol and 5% (w/v) urea in water, and 0·2 ml of a hypobromite solution prepared by dissolving 0·66 ml of Br_2 in 100 ml of 5% (w/v) KOH solution. The hypobromite solution was added quickly by blowing in with a 0·2 ml micropipette. The contents were mixed immediately by vigorous shaking. The tube was placed in an ice-bath for 10 min for the complete development of the pink colour. The tubes were removed, allowed to warm to room temperature, and the optical density of the solution was measured at 490 mμ.

RESULTS

The chromatographic procedure in these experiments was designed to separate arginine and histamine from glycocyamine and histidine. It takes less than 4 hours to perform the separation, so that the samples can be analysed on the same day that the

animals are killed. To check the separation of the basic amino acids by this procedure trichloroacetic acid filtrates of brain were chromatographed on the column in the usual way, and the eluates prepared for paper chromatography. Phenol-water-NH_3 and collidine (DENT, 1948) were the solvents used in two dimensions, and the paper was sprayed with a solution of ninhydrin. The main components in the fractions eluted by the citrate buffer were aspartic acid, glutamic acid, alanine, the leucines, and valine. As already described, histidine was found in tube 5 and glycocyamine in tubes 4 to 6. The fractions eluted by sodium hydroxide contained histamine, arginine, lysine, ethanolamine, glutamine, and substances giving two unidentified spots, one in the position of glucosamine and the other running faster than histidine in phenol. Added histidine, histamine, and arginine could be recovered quantitatively from the columns in amounts up to 100 μg.

The identity of the histamine separated in this way was confirmed pharmacologically. Samples of the eluate fractions were tested with the guinea-pig ileum by the usual pharmacological technique. These fractions produced responses indicating concentrations of histamine of 2·9 to 6·8 μg as compared with colorimetric estimates of 3·1 to 3·8 μg of histamine for the same samples. The pharmacological responses were abolished by the addition of histamine inhibitors.

TABLE 1.—THE EFFECT OF DIFFERENT EXPERIMENTAL PROCEDURES ON THE LEVELS OF HISTIDINE, HISTAMINE, AND ARGININE IN THE RAT BRAIN

Conditions	Histidine (μg/g)	Histamine (μg/g)	Arginine (μg/g)
Series (a) Unanaesthetized			
Control	26·1 ± 2·8	4·3 ± 1·0	10·9 ± 3·0
Insulin coma	23·2 ± 2·8	3·5 ± 1·5	9·8 ± 3·2
Series (b) Anaesthetized with sodium amytal before killing			
Control	25·6 ± 4·1	6·4 ± 2·5	14·2 ± 2·2
Insulin coma	26·7 ± 4·6	7·5 ± 0·8	8·5 ± 1·8
Exercise	25·9 ± 1·8	5·8 ± 1·8	13·2 ± 1·2
Chilling	26·9 ± 1·9	6·8 ± 2·4	11·9 ± 3·2

The mean values in each group were obtained by separate determinations on six animals. Values are expressed as μg/g fresh brain tissue ± standard deviation.

The values found for the histidine, histamine, and arginine content of the brain in normal unanaesthetized animals are shown in the series (a) control group in Table 1. No significant change in the free histidine in the brain was produced by anaesthesia with sodium amytal. Values about 11 % below the normal were obtained in the animals in insulin coma, but the difference was not significant ($P = 0·2$). The values obtained for histamine and arginine were also lower in insulin coma. The rats killed under anaesthesia (series b), gave slightly higher values for arginine ($P = 0·1$) and histamine ($P = 0·2$) in the brain, but again the differences were not significant. The only statistically significant experimental change was a decrease in the amount of arginine in the hypoglycaemic group in comparison with that in the control group ($P = 0·001$).

Using the Na-form of Zeo-Karb resin for the adsorption of arginine, the amount of glycocyamine in rat brain was found to be 16·1 ± 2·3 S.D. μg/g of fresh tissue. By the same procedure the level of free glycocyamine in kidney extracts was 38·8 ± 2·1 S.D. μg/g.

DISCUSSION

Histidine and arginine. Normal values for the free histidine and arginine in the rat brain were first obtained by SCHURR *et al.* (1950a, b), using a microbiological method of assay. WILLIAMS *et al.* (1950), who used the same method, found somewhat higher values (Table 2); the difference was attributed to their using rats of the Holtzman strain instead of the Sprague-Dawley rats used by SCHURR *et al.* Values for the free histidine and arginine in a cat brain were reported by TALLAN *et al.* (1954), who used a method of column chromatography. They were unable to detect either of the methyl histidines at the level of 3 μg/g of brain, which appears to be the lower limit of their method. All the values hitherto reported have been obtained on anaesthetized animals, and no special precautions were taken to prevent changes due to the action of proteinases, transaminases, or other enzymes during the removal of the organs. Since it is now known that anaesthesia changes the distribution of free amino acids in the brain (DAWSON, 1953; ANSELL and RICHTER, 1954), it appeared doubtful if the reported values should be accepted as normal.

TABLE 2.—COMPARISON OF VALUES FOUND BY DIFFERENT WORKERS FOR THE HISTIDINE AND ARGININE CONTENT OF THE BRAIN

The values give μg/g fresh brain ± S.D. or S.E.M. The animals were anaesthetized before killing

Species	Method	Histidine (μg/g)	Arginine (μg/g)	Reference
Rat (Sprague-Dawley)	Microbiological	8·6 ± 1·0 S.E.M.	22·3 ± 2·0 S.E.M.	SCHURR *et al.* (1950)
Rat (Holtzman)	Microbiological	17·4 ± 1·7 S.E.M.	33·8 ± 0·9 S.E.M.	WILLIAMS *et al.* (1950)
Cat	Chromatographic	9	14	TALLAN *et al.* (1954).
Rat (Wistar)	Colorimetric	25·6 ± 4·1 S.D.	14·2 ± 2·2 S.D.	Present investigation

In the present investigation, unanaesthetized Wistar albino rats were used: the brains were rapidly fixed by freezing in liquid oxygen and the histidine and arginine were determined by colorimetric methods after a preliminary separation on a resin column. It was shown that the levels of these amino acids were not significantly affected by anaesthesia with sodium amytal. The values obtained were of the same order as those obtained by the earlier workers using different methods: in so far as they differed from the earlier values, this might be due to the use of different strains of rats or to the measures taken to avoid post mortem change.

The present experiments gave little evidence of a participation of unbound histidine or arginine in the metabolic changes occurring during physiological activity of the brain. The free histidine content was remarkably constant in the rat brain, and it did

not vary significantly with any of the experimental procedures that were applied. The arginine content was less constant, and lower values were obtained in the brain in rats subjected to insulin coma.

Histamine. Histamine has been detected in nervous tissues by several workers who used pharmacological methods (KWIATKOWSKI, 1943). Quantities found in whole brain were usually of the order of 0·1 μg/g of fresh tissue, but in some areas the concentration was as high as 56 μg/g (MCILWAIN, 1955). By a method of pharmacological assay depending on the amount of inhibition of histamine activity by benadryl, CICARDO and STOPPANI (1949) found much higher values from 9·8 to 17·3 μg of free histamine/g in the dog brain.

The level of 4 μg/g found in the rat brain in the present study by chemical methods of estimation agreed closely with independent determinations by pharmacological assay with the guinea-pig ileum. Since the assays were performed on fractions purified by adsorption on chromatographic columns, the presence of compounds with histamine-like activity, or with anti-histamine activity, in the original brain extracts should not interfere with the assay, unless their physical properties were so similar to those of histamine that they occurred in the same eluate fraction. In this connection it may be noted that pharmacological assays on the whole trichloroacetic acid filtrate from brain gave values higher than those obtained by the chemical method; but with these extracts the response was only partially inhibited by histamine inhibitors. This suggests that substances other than histamine, with histamine-like activity, are present in the whole extract from brain.

Glycocyamine. The presence of glycocyamine in tissue extracts was previously reported by BORSOOK and DUBNOFF (1941). They found that the rat brain contained 30–60 μg of glycocyamine/g tissue. The somewhat lower levels of 13–19 μg/g were obtained by the methods used in the present investigation.

SUMMARY

(1) A method is described for the colorimetric determination of histidine and histamine in tissue extracts after a preliminary chromatographic separation on a resin column. By the same procedure, arginine is separated from glycocyamine. Arginine and glycocyamine may then be estimated colorimetrically by the Sakaguchi method.

(2) The normal level of histidine present in the free form in the rat brain is 26·1 $\pm$ 2·8 S.D. μg/g of fresh brain, of histamine 4·3 $\pm$ 1·0 S.D. μg/g, of arginine 10·9 $\pm$ 3·4 S.D. μg/g, and of glycocyamine 16·1 $\pm$ 2·3 μg/g.

(3) The effect of the physiological state on these brain constituents was studied. In anaesthetized, exercised, or chilled rats, the levels of arginine, histidine, and histamine in the rat brain did not vary significantly. In insulin hypoglycaemia the arginine content of the rat brain was significantly reduced ($P = 0{\cdot}001$), but the histidine and histamine content showed no significant change.

Acknowledgements—The authors wish to thank DR J. D. P. GRAHAM of the Welsh National School of Medicine through whose courtesy the pharmacological assays were obtained. They thank the U.S. Public Health Service for a fellowship from the National Institute of Neurological Disease and Blindness (D. H. C.) and the Rockefeller Foundation and the Medical Research Council for grants.

VOL.
1
1956/5

REFERENCES

ANSELL G. B. and RICHTER D. (1954) *Biochem. J.* **57,** 70.

BORSOOK H. and DUBNOFF J. W. (1941) *J. biol. Chem.* **138,** 389.

CICARDO V. H. and STOPPANI A. O. M. (1949) *Nature* Lond. **163,** 365.

DAWSON R. M. C. (1953) *Biochim. biophys. Acta.* **11,** 548.

DENT C. E. (1948) *Biochem. J.* **43,** 169.

DUBNOFF J. W. (1941) *J. biol. Chem.* **141,** 711.

FRIEDBERG F. and GREENBERG D. M. (1947) *J. biol. Chem.* **168,** 411.

GAITONDE M. K. and RICHTER D. (1955) *Biochem. J.* **59,** 690.

GAITONDE M. K. and RICHTER D. (1956) *Proc. Roy. Soc.* **B145,** 83.

KWIATKOWSKI H. (1943) *J. Physiol.* **102,** 32.

MACPHERSON H. T. (1942) *Biochem. J.* **36,** 59.

MCILWAIN H. (1955) *Biochemistry of the Nervous System* p. 218. Churchill, London.

SCHURR P. E., THOMPSON H. T., HENDERSON L. M., and ELVEHJEM C. A. (1950a) *J. biol. Chem.* **182,** 29.

SCHURR P. E., THOMPSON H. T., HENDERSON L. M., WILLIAMS J. N. JR., and ELVEHJEM C. A. (1950b) *J. biol. Chem.* **182,** 39.

TALLAN H. H., MOORE S., and STEIN W. H. (1954) *J. biol. Chem.* **211,** 927.

TALLAN H. H. (1956) *Fed. Proc.* **15,** 368.

VRBA R. (1956) *J. Neurochem.* **1,** 12.

WILLIAMS J. N. JR., SCHURR P. E., and ELVEHJEM C. A. (1950) *J. biol. Chem.* **182,** 55.

OL.
1
6/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 234-244 Pergamon Press Ltd., London

QUANTITATIVE DETERMINATION OF CHOLINESTERASE IN INDIVIDUAL SYMPATHETIC CELLS

EZIO GIACOBINI
Department of Physiology, Karolinska Institutet, Stockholm

(*Received* 11 *October* 1956)

VOL
1
1956/

THE cholinesterase activity of the sympathetic ganglia has been the subject of several investigations since GLICK (1937) found that both nonspecific ChE* and AChE are present in the superior cervical ganglion and suggested that the ChE activity may be concentrated at the synapses. The ChE activity of these ganglia has been further studied by BRÜCKE (1937), NACHMANSOHN (1937; 1938), CROXATTO, HUIDOBRO, SALVESTRINI, DONOSO, SANHUEZA, and LUCO (1940), MENDEL and RUDNEY (1944). GLICK's results were confirmed by SAWYER and HOLLINSHEAD (1945), who, employing extracts of almost the entire ganglia, found both ChEs. When studying the distribution of ChE in the nervous system by the analysis of homogenates, it is impossible to distinguish whether the enzyme is concentrated in a small number of neurones or whether it is present in smaller amount in a large number of cells. Further, it has been shown that non-nervous tissue contains a considerable amount of the nonspec. ChE (SAWYER, 1946; HARD and PETERSON, 1950; HARD, PETERSON, and FOX, 1951; KOELLE, 1951–1952; HEBB, SILVER, SWAN, and WALSH, 1953; THOMPSON and TICKNER, 1953; CAVANAGH, THOMPSON, and WEBSTER, 1954). It is therefore difficult to interpret the results of a chemical analysis of gross specimens of tissue and to correlate them with the activity of single nerve cells or their component parts. Several histochemical techniques have been applied to overcome this difficulty (GOMORI, 1948; KOELLE and FRIEDENWALD, 1949; BARNETT and SELIGMAN, 1951; HOLT and WITHERS, 1952; RAVIN, ZACKS, and SELIGMAN, 1953; CREVIÈR and BELANGÈR, 1955). Nearly all these methods are subject to serious diffusion artefacts. In the region of a structure of high enzymic activity, it is difficult to know whether the area in which the precipitation product of the histochemical reaction is seen, represents the actual location of the enzyme. For the same reason it is also difficult to use the histochemical method for the demonstration of the enzyme in structures with low activity and therefore requiring a prolonged incubation. These methods provide information about the relative ChE activity in the different cells shown in the sections, but no conclusions can be drawn from them about the localization of the enzyme in single cells.

In an earlier investigation the author applied a modification of the thiocholine method (ZAJICEK, SYLVÉN, and DATTA, 1954) to single nerve cells isolated from fresh tissue by micromanipulation, to study the distribution of AChE within the cell body and along the axon (GIACOBINI, 1956). In neurones of the spinal, sympathetic, and

* In this paper the symbol ChE (cholinesterase) signifies all enzymes splitting choline esters, irrespective of their specificity.

AChE = acetylcholinesterase, true cholinesterase, specific cholinesterase, erythrocyte cholinesterase, aceto-cholinesterase, cholinesterase-I. Nonspec. ChE = nonspecific cholinesterase, pseudo-cholinesterase, serum cholinesterase, butyro- and propiono-cholinesterase, cholinesterase-II.

parasympathetic ganglia, as well as in cells of the anterior and the lateral horns of the spinal cord (frog, rat, and cat), large concentrations of ChE could be demonstrated in the cytoplasm and the neurite after a short incubation. The relative number of such ChE-bearing cells was found to be considerably larger than in previous investigations.

In order to study the topical distribution of the enzyme it was necessary to determine the ChE activity of single structural units by more sensitive techniques. From the data available on ChE activity of a whole sympathetic ganglion, it can be estimated that the amount of CO_2 liberated from a single neurone during the ester hydrolysis is of the order of 10^{-4} to 10^{-2} μl CO_2/hour, corresponding to the hydrolysis of about 0·02 to 2 μg acetylthiocholine. The determination of ChE activity at such levels is not possible by the usual biochemical methods. The smallest amount of CO_2 that can be measured by the usual manometric methods is about 1–5 μl CO_2/hour (Warburg apparatus). Even the most sensitive micromethods hitherto used for following the respiration of unicellular micro-organisms, such as single egg cells, protozoa, syncitia, etc., have a sensitivity too low for estimations on single somatic cells.

In 1937, LINDERSTRÖM-LANG introduced a microgasometric method based on the principle of the Cartesian diver, and in 1938 LINDERSTRÖM-LANG and GLICK developed a Cartesian diver method for the measurement of ChE based on the principle, utilizing the Warburg apparatus (HAMMON, 1933). Since it was first described, the diver technique has been greatly developed. Several variations of the original form have been introduced (HOLTER and LINDERSTRÖM-LANG, 1938; LINDERSTRÖM-LANG and GLICK, 1938; BOELL, NEEDHAM, and ROGERS, 1939; BOELL, KOCH, and NEEDHAM, 1939; ROCHER, 1942; HOLTER, 1943; ZEUTHEN, 1943–1953). The method has also been successfully applied to the analysis of the ChE activity of small samples of serum and of gastric mucosa (LINDERSTRÖM-LANG and GLICK, 1938), extracts of embryonic nervous tissue (BOELL and SHEN, 1944), and homogenates of fresh and frozen-dried preparations of nerves and brain (BOELL and NACHMANSOHN, 1940; BOELL, 1945–1955). A further modification of the diver technique introduced by ZAJICEK and ZEUTHEN (1956) has been applied by ZAJICEK for determining the AChE activity of single megacaryocytes of the bone marrow. By this technique it has been possible to reach a sensitivity of 10^{-4} μl CO_2 with 10% accuracy.

The present investigation deals with the application of the microdiver technique for the determination of the ChE and AChE activity of single neurones and their constituent parts (neurite, cell body) isolated from sympathetic ganglia of frog and rat by micromanipulation. Some findings on the inter- and intracellular distribution of ChE in ganglion cells are also reported.

MATERIAL AND METHODS

A. *Microdissection technique*

The experiments were performed on frogs (*Rana temporaria*), and albino rats weighing about 200 g. The animals were killed by decapitation with or without light ether anaesthesia. A sympathetic ganglion of the animal was quickly dissected out, placed in the moist, oxygenated chamber of the microdissection instrument, and immersed in a drop of substrate-bicarbonate Ringer solution. Individual nerve cells were further dissected, and from these, single cell bodies and 100–300 μ long parts of neurite were separated by micromanipulation, as previously described (GIACOBINI, 1956). Care was taken that the cell bodies obtained in this way were completely free from the surrounding connective tissue capsule and capsule cells (Fig. 3A). The cell bodies and the isolated fibres were

then examined at high magnification in phase contrast, and photographed. Their diameters were measured by means of a Zeiss ocular screw micrometer (measurement was effected at 800 linear magnifications). Cells or fibres which on microscopic inspection were found to be injured, were discarded.

B. *Cartesian diver technique*

The enzyme activity was expressed as the amount of carbon dioxide evolved from the bicarbonate buffer by the acetic acid formed during the enzymic hydrolysis of AThCh (acetylthiocholine). The part of the neurone to be analysed was sucked into the diver, an open glass vessel with a gas volume of 0·2–0·5 μl and weighing about 1–1·5 mg (Figs. 2 and 3). The substrate-bicarbonate solution

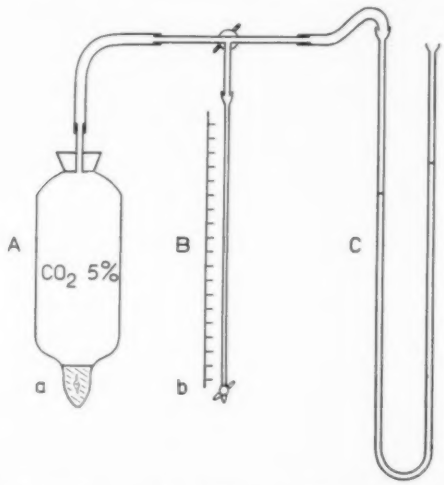


FIG. 1.—Arrangement of the Cartesian diver apparatus. (For explanation of the letters see text.)

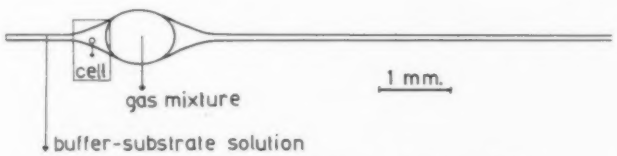


FIG. 2.—Schematic representation of a charged diver.

and a small quantity of 5% CO_2/95% N_2 had previously been introduced into the diver. The substrate used was a $6{\cdot}5 \times 10^{-3}$ M solution of AThCh (acetylthiocholine iodide, HOFFMANN-LA ROCHE) saturated with 5% CO_2/95% N_2 mixture (pH *ca.* 7·5) (ZAJICEK and ZEUTHEN, 1956). The incubation medium was prepared by dissolving 34 mg AThCh in a solution made by adding 10 ml 0·8% NaCl to 10 ml 0·28% $NaHCO_3$ and 0·4 ml 1·34% $CaCl_2.H_2O$ (total volume 20·4 ml).

After the diver had been charged with the buffer-substrate solution and the cell fragment (Fig. 1), it was closed with wax at one end and placed in the flotation medium contained in vessel *A*, which was immersed in a water-bath maintained at constant temperature (25 $\pm$ 0·001°). The vessel was connected to a micromanometer *B*, and by means of a screw *b* it was possible to adjust the pressure in *A* so as to make the diver float at a given level. The position of the diver was read with a travelling microscope. The change in equilibrium pressure due to the development of CO_2 in the diver was measured at 15-min intervals during a period of 4–8 hours, and from it the change in volume of the gas-bubble, expressing the ChE activity, curve could be determined.

VOL.
1
56/57

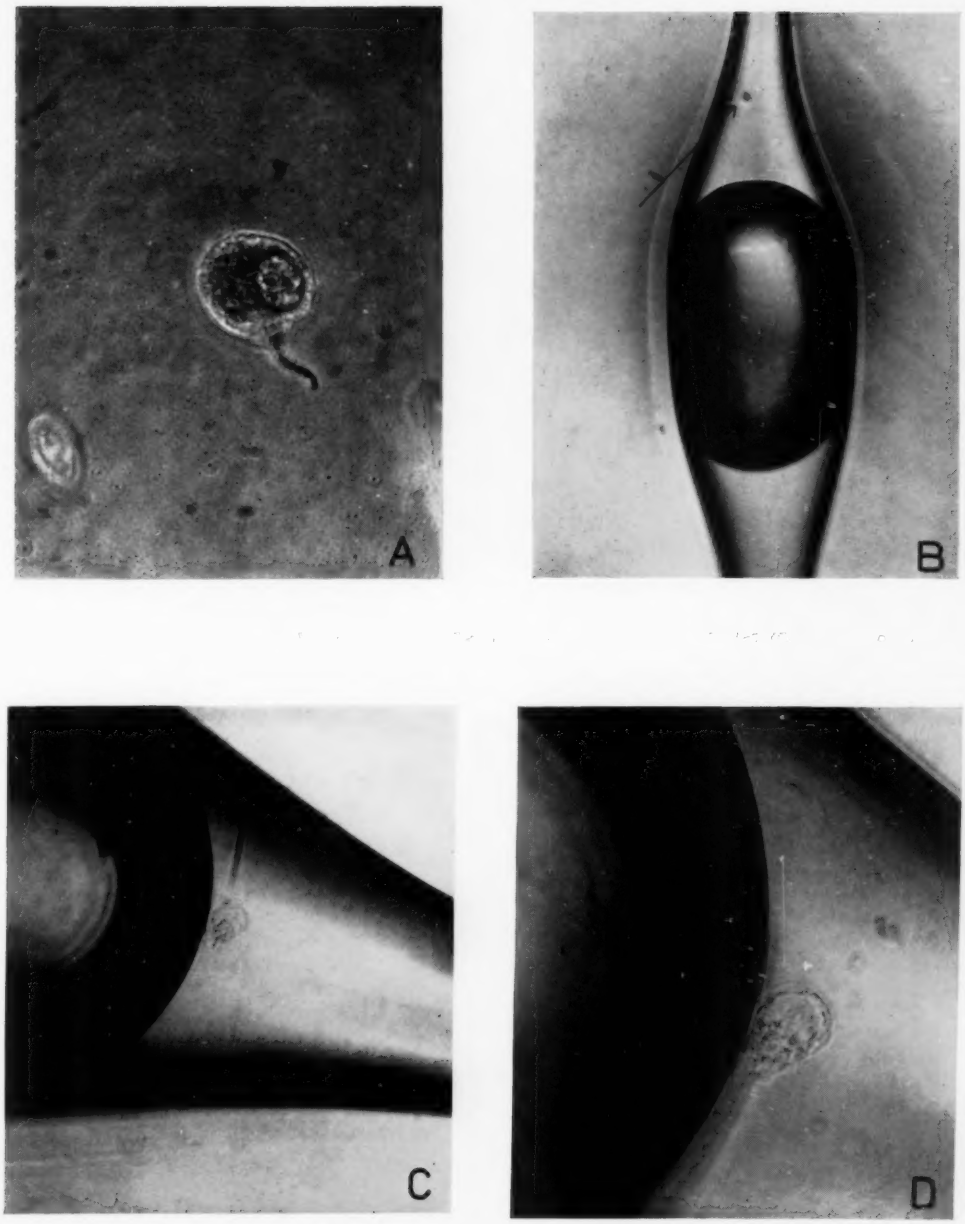


FIG. 3.—*A*. A nerve cell isolated from a sympathetic ganglion of the rat, suspended in Ringer-bicarbonate solution.
B. The same cell placed in the diver.
C and *D*. A cell body (30 μ) isolated from a rat sympathetic ganglion is in the diver shown at two different magnifications.

VOL
1
1956/

The gas exchanged per unit time $\Delta CO_2/\Delta t$ given in Tables 1, 2, and 3 was calculated according to the following formula deduced by ZEUTHEN (1953) and ZAJICEK and ZEUTHEN (1956):

$$\frac{\Delta CO_2}{\Delta t} = \frac{v(B + h - e)[273\ V_G + (273 + t^\circ)\alpha_{CO_2} V_F]}{10300\ V\ (273 + t^\circ)}\ x$$

where:

x = movement in mm per unit time in the capillary micromanometer B(1 mm = 0·8 μl = v).
v = volume in μl per mm of the capillary (manometer B).
V = volume of the gas phase of the manometer B (μl).
V_G = gas volume of the floating diver.
B = barometric pressure expressed in mm H_2O (760 mm Hg ~ 10,300 mm H_2O).
h = initial equilibrium pressure of the diver read on C (mm H_2O).
e = vapour tension of water in mm H_2O (25°).
α_{CO_2} = absorption coefficient for CO_2 (25°).
V_F = volume of the fluid enclosed in the diver (μl).

In order to differentiate between AChE and nonspec. ChE, a selective inhibitor, mipafox (bis-aminophosphoryl iodide), was used. As has recently been demonstrated (ALDRIDGE, 1953; DAVISON 1953; HOLMSTEDT, 1957), this substance completely inhibits the nonspec. ChE of human serum at a concentration of 8×10^{-7} M, whereas it does not affect the AChE of human erythrocytes at a concentration lower than 6×10^{-6} M. Controls performed on the ChE of the rat brain and spinal cord confirmed that 5×10^{-6} M-mipafox did not inhibit the AChE, while non-spec. ChE activity was completely inhibited (DAVISON, 1953; GIACOBINI and HOLMSTEDT, 1956).

The quantitative determination of ChE and AChE per nerve cell was carried out by the following procedure (GIACOBINI and ZAJICEK, 1957):

(1) determination of the total cholinesterase activity by the hydrolysis of AThCh.

(2) determination of the AChE activity after inhibition of the nonspec. ChE by the introduction into the diver of a known quantity of a 5×10^{-6} M-solution of mipafox (GIACOBINI, 1957)

Parallel control experiments were carried out with a known quantity of human serum ChE and finally with a sample of the fluid in which the cell was suspended (Figs. 4 and 5).

RESULTS

The esterase activity curves of a cell body (33 μ diameter) isolated from a sympathetic ganglion of a rat and of a 95 μ long part of its neurite and finally of the buffer-substrate Ringer solution in which the cell was suspended, are given in Fig. 4. The graph shows that the cell body liberated about $18{\cdot}5 \times 10^{-4}$ μl CO_2/hour and the 95 μ long part of the neurite 1×10^{-4} μl CO_2/hour, corresponding to the hydrolysis of about $21{\cdot}5 \times 10^{-3}$ μg AThCh and $1{\cdot}1 \times 10^{-3}$ μg AThCh respectively.

Fig. 5 shows the esterase activity curves of a cell body (25 μ) isolated from a sympathetic ganglion of rat and of a 110 μ long part of its neurite, and finally of a sample of purified serum ChE before and after treatment with mipafox. Lastly, the same graph shows the activity curve of a control buffer solution. The graph shows that the cell body and its neurite segment liberated about $19{\cdot}8 \times 10^{-4}$ μl and $0{\cdot}8 \times 10^{-4}$ μl CO_2/hour respectively, corresponding to the hydrolysis of about $23{\cdot}1 \times 10^{-3}$ μg AThCh and $0{\cdot}9 \times 10^{-3}$ μg AThCh/hour respectively. The cell body and the neurite exhibited the same esterase activity after treatment with mipafox as before. It can therefore be concluded that the esterase found in this cell was AChE.

Table 1 shows the ChE and AChE activity, expressed in μl CO_2/hour of single cell bodies dissected from sympathetic ganglia of the frog. The values reported show that a wide variation exists in the ChE activity of cells belonging to the same ganglion or to the same type of ganglion. These cells show a variation from about 2×10^{-4} μl CO_2/hour to 60×10^{-4} μl CO_2/hour in ChE activity. The cells giving low values

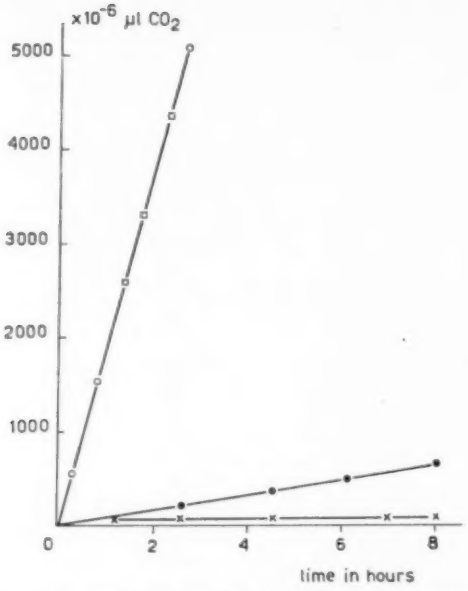


FIG. 4.—AChE activity curve of a cell body (33 μ) isolated from a sympathetic ganglion of the rat (○); that of a 95 μ long part of its neurite (●); and that of the buffer-substrate Ringer's solution in which the cell was suspended (×).

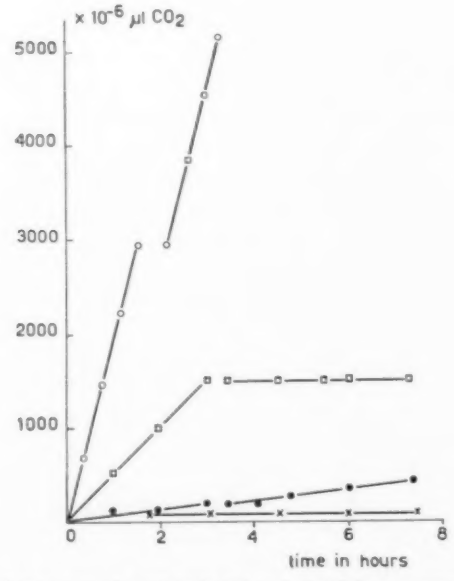


FIG. 5.—AChE activity curve of a cell body (25 μ) isolated from a sympathetic ganglion of the rat (○), that of 110 μ long part of its neurite (●), of a sample of purified ChE (□), and of a control buffer-substrate solution (×).

TABLE 1.—CHOLINESTERASE ACTIVITY OF SINGLE INTACT NERVE CELL BODIES FROM SYMPATHETIC GANGLION OF THE FROG*

Dry weight of diver (mg)	Cell diam. (μ)	ChE activity (μl CO_2/hr)	Sympathetic ganglion (frog)
2·54	35	$+61{\cdot}5 \times 10^{-4}$	L I
1·77	40	$35{\cdot}0 \times 10^{-4}$	L I
3·28	45	$29{\cdot}8 \times 10^{-4}$	L I
1·3	26	$24{\cdot}8 \times 10^{-4}$	L II
1·58	36	$12{\cdot}1 \times 10^{-4}$	L I
1·51	45	$11{\cdot}2 \times 10^{-4}$	L II
1·12	31	$+10{\cdot}9 \times 10^{-4}$	L III
1·79	41	$10{\cdot}8 \times 10^{-4}$	L II
1·21	25	$+\ 9{\cdot}3 \times 10^{-4}$	L IV
2·45	20	$7{\cdot}7 \times 10^{-4}$	L I
1·57	38	$4{\cdot}1 \times 10^{-4}$	L I
1·63	24	$+\ 1{\cdot}9 \times 10^{-4}$	L I
—	26	no meas. activity	L II
—	31	no meas. activity	L I
—	42	no meas. activity	L IV
—	30	no meas. activity	L I
—	29	no meas. activity	L I
—	36	no meas. activity	L I

* In Tables 1, 2, and 3 the sign + indicates a figure expressing AChE activity. The activity values are given in order of decreasing activity.

represent the majority of the cell population of the ganglion, while the cells showing the highest values are very rare. In a certain number of cells no measurable activity could be demonstrated.

Table 2 gives the ChE activity of single segments from the initial part of the axon isolated from postganglionic sympathetic fibres of the frog (mean thickness about 2·6 μ; length varying from 120 to 320 μ). Even in these structures the ChE and AChE activity varied widely. In some neurites very high values were found, but in the majority only a moderate or low activity could be demonstrated. Finally, in a certain number of neurites no ChE activity could be demonstrated with the technique employed. The ChE and AChE activity was determined also in the cell body and in the initial segment of the axon of the same cell. The results in Table 3 (sympathetic ganglia of rat) show that the distribution of enzymes seems to be comparable to that in the frog. The AChE activity of the cell body was often similar to that of the respective neurite, but in some cells this did not apply. Tables 1, 2, and 3 show that the esterase found in a high proportion of cells was AChE.

DISCUSSION

The ChE activity of the sympathetic ganglia has been the subject of several studies since GLICK (1937; 1938) suggested that this enzyme may be concentrated at the synapse. In the superior cervical ganglion of the cat both ChEs were found in high and almost equal concentrations. BRÜCKE (1937) found a marked decrease of the ChE

TABLE 2.—CHOLINESTERASE ACTIVITY OF SEGMENTS FROM SINGLE POSTGANGLIONIC SYMPATHETIC FIBRES OF THE FROG (MEAN THICKNESS ABOUT 2·6 μ)

Dry weight of the diver (mg)	Axon length (μ)	ChE activity (μl CO_2/hr)
1·28	250	$22{\cdot}5 \times 10^{-4}$
1·47	260	$+14{\cdot}4 \times 10^{-4}$
1·0	280	$9{\cdot}46 \times 10^{-4}$
2·18	240	$+7{\cdot}98 \times 10^{-4}$
0·93	310	$+5{\cdot}95 \times 10^{-4}$
1·9	320	$4{\cdot}10 \times 10^{-4}$
1·47	140	$3{\cdot}23 \times 10^{-4}$
1·64	120	$2{\cdot}58 \times 10^{-4}$
1·14	220	$+2{\cdot}50 \times 10^{-4}$
1·91	280	$+2{\cdot}11 \times 10^{-4}$
1·39	180	$1{\cdot}60 \times 10^{-4}$
1·77	150	$+1{\cdot}45 \times 10^{-4}$
1·3	165	$1{\cdot}24 \times 10^{-4}$
—	195	no meas. activity
—	218	no meas. activity
—	185	no meas. activity
—	110	no meas. activity
—	140	no meas. activity
—	95	no meas. activity

content of this ganglion after cutting the preganglionic fibres. COUTEAUX and NACHMANSOHN (1942) found that a ganglion in which the preganglionic fibres had been cut twelve days earlier, still contained 40% of its original ChE activity. SAWYER and HOLLINSHEAD (1945) found that, after section of the preganglionic fibres, the AChE activity in the ganglion falls after the first week to 25% and the nonspec. ChE activity to 75% of the original level.

These different investigations indicate that AChE must accumulate in the region where the preganglionic endings and ganglion cells are concentrated. Section of the fibres afferent to, or efferent from, the ganglion may, however, produce a large and unspecific damage to the structure of the ganglion. The fact that the esterase activity in these investigations was estimated in extracts of almost whole ganglia also makes it difficult to interpret the results. The present work (Tables 1, 2, and 3) shows that a relatively high proportion of sympathetic ganglion cells contain AChE (GIACOBINI, 1956).

The values reported show a wide variation in ChE activity, but in this respect against what has been indicated by earlier histochemical work (KOELLE, 1951–1955; GIACOBINI, 1956); ganglion cells can hardly be classified in groups of cells of uniform type. The cells exhibiting the highest ChE activity (5–30 times higher than the rest) seem to be scarce, and the majority of the cells show an activity of about 2×10^{-4}–10 μl CO_2/hour. Finally, in a certain number of cells no activity could be demonstrated, even if the measurement of the CO_2 evolution was prolonged to 8 hours. In these cells

TABLE 3.—CHOLINESTERASE ACTIVITY OF SINGLE NERVE CELLS FROM SYMPATHETIC GANGLION OF THE RAT

Dry weight of diver (mg)	Cell diam. (μ)	Cell body activity (μl CO_2/hr)	Axon length (μ) (m thickness 2·2 μ)	Axon activity (μl CO_2/hr)
1·63	35	$+31{\cdot}1 \times 10^{-4}$	145	$+10{\cdot}9 \times 10^{-4}$
1·80	25	$21{\cdot}0 \times 10^{-4}$	170	$9{\cdot}95 \times 10^{-4}$
0·98	40	$+19{\cdot}4 \times 10^{-4}$	190	$8{\cdot}81 \times 10^{-4}$
0·99	36	$18{\cdot}0 \times 10^{-4}$	205	$8{\cdot}00 \times 10^{-4}$
1·30	24	$+14{\cdot}6 \times 10^{-4}$	195	$+6{\cdot}6 \times 10^{-4}$
1·05	25	$11{\cdot}3 \times 10^{-4}$	185	$+7{\cdot}05 \times 10^{-4}$
0·95	45	$+ 9{\cdot}4 \times 10^{-4}$	210	$+6{\cdot}91 \times 10^{-4}$
1·31	40	$9{\cdot}1 \times 10^{-4}$	270	$7{\cdot}64 \times 10^{-4}$
1·92	35	$8{\cdot}2 \times 10^{-4}$	190	$5{\cdot}46 \times 10^{-4}$
1·76	40	$+7{\cdot}6 \times 10^{-4}$	135	$+4{\cdot}37 \times 10^{-4}$
1·21	25	$4{\cdot}3 \times 10^{-4}$	195	$2{\cdot}19 \times 10^{-4}$
1·91	48	$4{\cdot}1 \times 10^{-4}$	220	$3{\cdot}11 \times 10^{-4}$
1·56	38	$+3{\cdot}9 \times 10^{-4}$	300	$+3{\cdot}26 \times 10^{-4}$
1·31	45	$+3{\cdot}6 \times 10^{-4}$	195	$+1{\cdot}43 \times 10^{-4}$
1·34	30	$3{\cdot}4 \times 10^{-4}$	160	$1{\cdot}21 \times 10^{-4}$
0·90	27	$2{\cdot}4 \times 10^{-4}$	180	$2{\cdot}10 \times 10^{-4}$
0·78	29	$2{\cdot}1 \times 10^{-4}$	175	$1{\cdot}06 \times 10^{-4}$
1·06	22	$1{\cdot}5 \times 10^{-4}$	135	$0{\cdot}96 \times 10^{-4}$
—	40	no meas. activity	—	no meas. activity
—	38	no meas. activity	—	no meas. activity
—	26	no meas. activity	—	no meas. activity
—	25	no meas. activity	—	no meas. activity
—	21	no meas. activity	—	no meas. activity
—	18	no meas. activity	—	no meas. activity

the enzyme activity must be absent or below the limit of sensitivity of the method employed. These ganglion cells are believed to be of the same type as those in which no AChE activity can be demonstrated by the histochemical methods after an incubation period of 24 hours.

The AChE distribution pattern found in the sympathetic ganglion raises the question of whether these different levels of activity in the various cells represent different stages in the synthesis of the enzyme, whether they are an expression of varying function, or whether they represent different types of cells.

From studies on the distribution of ChE in the nervous system at different stages of development (SAWYER, 1943), it is known that ChE is present in nervous tissues in the early premotile embryonic stages, that its concentration increases rapidly as soon as nervous function begins, and that it increases progressively with the differentiation of the nervous tissue. In sheep embryos the esterase activity of the spinal cord increases in parallel with the appearance of reflex muscular movements (NACHMANSOHN, 1940). Using histochemical methods, ZACKS (1954) traced the development of esterase activity through the very early stages in the chick embryo, and showed that AChE activity can be detected in the developing nervous system before the morphological

differentiation of the neuroblasts occurs. Very recently, ZAJICEK (1956) studied the increase of cholinesterase activity during the maturation of the megacaryocytes. He has also found that megacaryocytes of an apparently similar degree of maturation exhibited large variations (up to 5%) in the enzyme content. He discussed the possibility that during the process of maturation, in order to reduplicate themselves, the active centres of cholinesterase might combine with other molecules (e.g. ribonucleic acid), with a resulting temporary inactivation of the enzyme in the cell. This process is of great interest, since it has been shown by HYDÉN (1943) and EDSTRÖM and HYDÉN (1954) that a considerable amount of ribonucleic acid is present in the cytoplasm of the nerve cell; this is believed to take an active part in the different functional phases of the cell. The process of synthesis of AChE is, however, not sufficiently known to permit any conclusion to be drawn.

The particular distribution of the enzyme found in sympathetic cells suggests the possibility that the difference in AChE activity may correspond to cells of different functional nature, *i.e.* the cell population of a sympathetic ganglion may not be pharmacologically homogeneous. Pharmacological work has shown that a certain number of postganglionic sympathetic fibres are probably of cholinergic nature, among them the vaso-dilator fibres of the cervical sympathetic, the hypogastric nerves, the fibres innervating the sweat glands, the vasodilator supplying the hind limb of the cat, and the lumbar sympathetic nerves of the dog. This hypothesis could give biochemical support to the theory proposed by SHAW *et al.* (1951). From a study of the action of several drugs (tetraethylammonium iodide, nicotine, curare, and antilusin) on the action potentials of the postganglionic fibres of the superior cervical ganglion, these authors suggested that it might contain a group of cells which do not respond in the same way as the rest to these paralysant drugs. On this basis, they put forward the theory of a dual pharmacological nature of the sympathetic ganglion cells.

These questions are linked with the intricate problem of the specific localization inside the nerve structures of the substances forming the chemical background to the propagation of the nerve impulse; possibly the application of quantitative microchemical methods in combination with electrophysiological techniques will provide a new experimental approach.

SUMMARY

A description is given of the application of the micro-diver technique to the determination of the ChE and AChE activity in single neurones and their constituent parts (cell body, neurite) isolated from sympathetic ganglia of the frog and rat by micromanipulation.

The values reported show a wide variation of enzymic activity from 2×10^{-4} to 30×10^{-4} μl CO_2/hour (rat). In this respect the sympathetic ganglion cells cannot be classified into uniform groups.

The findings are discussed in relation to the inter- and intracellular distribution of ChE in sympathetic ganglion cells.

Acknowledgement—This work has been supported by grants from The Swedish Medical Research Council.

VOL
1
1956/

REFERENCES

ALDRIDGE W. N. (1953) *Biochem J.* **53,** 62.
BARNETT R. J. and SELIGMAN A. M. (1951) *Science* **114,** 579.
BOELL E. J., KOCH H., and NEEDHAM J. (1939) *Proc. Roy. Soc.* **B 127,** 363.
BOELL E. J., NEEDHAM J., and ROGERS V. (1939) *Proc. Roy. Soc.* **B 127,** 356.
BOELL E. J., NEEDHAM J., and ROGERS V. (1939) *Proc. Roy. Soc.* **B 127,** 322.
BOELL E. J. and NEEDHAM J. (1939) *Proc. Roy. Soc.* **B 127,** 363.
BOELL E. J., KOCH H., and NEEDHAM J. (1939) *Proc. Roy. Soc.* **B 127**374.
BOELL E. J. and NACHMANSOHN D. (1940) *Science* **92,** 513.
BOELL E. J. and SHEN S. C. (1944) *J. exp. Zool.* **97,** 21.
BOELL E. J. (1945) *J. cell. comp. Physiol.* **25,** 75.
BOELL E. J. and SHEN S. C. (1949) *Anat. Rec.* **105,** 490
BOELL E. J. and SHEN S. C. (1950) *J. exp. Zool.* **113,** 583.
BOELL E. J. and SHEN S. C. (1951) *Anat. Rec.* **111,** 94.
BOELL E. J., GREENFIELD P., and SHEN S. C. (1955) *J. exp. Zool.* **129,** 415.
BRÜCKE F. T. V. (1937) *J. Physiol.* **89,** 429.
CAVANAGH J. B., THOMPSON R. M. S., and WEBSTER G. R. (1954) *Quart. J. exp. Physiol.* **39,** 185.
COUTEAUX R. and NACHMANSOHN D. (1940) *Proc. Soc. exp. Biol., N.Y.* **43,** 177.
COUTEAUX R. (1942) *Bull. Biol. France Belg.* **76,** 14.
CROXATTO H., HUIDOBRO R. F., SAEVESTRINI H., DONOSO R., SANHUEZA F., and LUCO I. V. (1940) *Ann. Acad. Biol. Univ. Chile* **3,** 7, 11, 15.
CREVIÈR M. and BELANGÈR L. F. (1955) *Science* **122,** 556.
DAVISON A. N. (1953) *Biochem J.* **54,** 583.
EDSTRÖM J. E. and HYDÉN H. (1954) *Nature* **174,** 128.
GIACOBINI E. and ZAJICEK J. (1956) *Nature* **177,** 185.
GIACOBINI E. (1956) *Acta physiol. scand.* **36,** 276.
GIACOBINI E. (1957) *Acta physiol. scand.* (to be published).
GIACOBINI E. and HOLMSTEDT B. (1957) *Acta physiol. scand.* (to be published).
GLICK D. (1937) *Nature* **140,** 426.
GLICK D. (1938) *C. R. Lab. Carlsberg, Ser. Chim.* **21,** 263.
GLICK D. (1938) *J. gen. Physiol.* **21,** 289.
GLICK D. (1939) *J. biol. Chem.* **130,** 527.
GOMORI G. (1948) *Proc. Soc. exp. Biol., N.Y.* **68,** 354.
HARD W. L. and PETERSON A. C. (1950) *Anat. Rec.* **108,** 57.
HARD W. L., PETERSON A. C., and FOX A. B. (1951) *J. Neuropath. exp. Neurol.* **10,** 48.
HEBB C. O., SILVER A., SWAN A. A. B., and WALSH E. G. (1953) *Quart. J. exp. Physiol.* **39,** 185.
HOLMSTEDT B. (1957) (to be published).
HOLTER H. and LINDERSTRÖM-LANG K. (1938) *C. R. Lab. Carlsberg, Ser. chim.* **22,** 300.
HOLTER H. (1943) *C. R. Lab. Carlsberg, Ser. chim.* **24,** 339.
HYDÉN H. (1943) *Acta physiol. scand.* Suppl. 17.
KOELLE G. B. and FRIEDENWALD J. S. (1949) *Proc. Soc. exp. Biol., N.Y.* **70,** 617.
KOELLE G. B. (1950) *J. Pharmacol.* **100,** 158.
KOELLE G. B. (1955) *J. Neuropath.* **14,** 23.
LINDERSTRÖM-LANG K. (1937) *Nature* **140,** 108.
LINDERSTRÖM-LANG K. and GLICK D. (1938) *C. R. Lab. Carlsberg, Ser. chim.* **22,** 300.
LINDERSTRÖM-LANG K. (1943) *C. R. Lab. Carlsberg, Ser. chim.* **24,** 333.
LINDERSTRÖM-LANG K. (1946) *C. R. Lab. Carlsberg, Ser. chim.* **25,** 229.
MENDEL B. and RUDNEY H. (1944) *Science* **100,** 499.
NACHMANSOHN D. (1937) *Nature* **140,** 427.
NACHMANSOHN D. (1938) *C. R. Soc. Biol.* **127,** 894.
NACHMANSOHN D. (1939) *Bull. Soc. chim. biol.* **21,** 761.
NACHMANSOHN D. (1940) *J. Neurophysiol.* **3,** 396.
NACHMANSOHN D. (1950) *Biochim. biophys. Acta* **4,** 78.
RAVIN H. A., ZACKS S. J., and SELIGMAN M. (1953) *J. Pharmacol.* **107,** 37.
SAWYER C. H. (1943) *J. exp. Zool.* **92,** 1.
SAWYER C. H. (1943) *J. exp. Zool.* **94,** 1.

SAWYER C. H. (1946) *Amer. J. Physiol.* **146,** 246.
SAWYER C. H. and HOLLINSHEAD W. H. (1945) *J. Neurophysiol.* **8,** 137.
SHAW F. H., KEOGH P., and MACCALLUM M. (1948) *Austr. J. exp. Biol.* **26,** 146.
SHAW F. H. and MACCALLUM M. (1949) *Austr. J. exp. Biol.* **27,** 296.
SHAW F. H., MACCALLUM M., DEWHURST D. J., and MAINLAND J. F. (1951) *Austr. J. exp. Biol.* **29,** 160.
THOMPSON R. H. S. and TICKNER A. (1953) *J. Physiol.* **121,** 623.
ZACKS G. I. (1954) *Anat. Rec.* **118,** 509.
ZAJICEK J., SYLVÉN B., and DATTA N. (1954) *J. Histochem. Cytochem.* **2,** 115.
ZAJICEK J. and ZEUTHEN E. (1956) *Exp. Cell. Res.* (in press).
ZAJICEK J. (1956) *Experientia* **6,** 226.
ZEUTHEN E. (1943) *C. R. Lab. Carlsberg, Ser. chim.* **24,** 479.
ZEUTHEN E. (1943) *Biol. Bull.* **188,** 366.
ZEUTHEN E. (1946) *C. R. Lab. Carlsberg, Ser. chim.* **25,** 191.
ZEUTHEN E. (1950) *Biol. Bull.* **98,** 139.
ZEUTHEN E. (1953) *Embryol. Exp. Morph.* **1,** 239.
ZEUTHEN E. (1955) *C. R. Lab. Carlsberg, Ser. chim.* **24,** 479.

VOL
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 245-249. Pergamon Press Ltd., London

THE OXYGEN UPTAKE OF THE PERIPHERAL NERVE OF THE RAT

P. F. CRANEFIELD,* F. BRINK,† and D. W. BRONK†
The Thomas C. Jenkins Laboratories of Biophysics
of the Johns Hopkins University, Baltimore

(*Received* 5 *January* 1956)

VOL. 1 56/57

THE oxygen metabolism of the peripheral nerve of mammals has been studied much less extensively than has that of the nerves of frogs and invertebrates. In particular the relation between frequency of nerve impulses and magnitude of the increased oxygen consumption of active nerve is not known. The results described below include the determination of the rate of oxygen uptake of rat's nerve at rest, evidence that the rate of oxygen uptake reaches a higher steady level when the nerve is conducting impulses, determination of these activity levels as a function of frequency, and a comparison of the effect of temperature on the rates of uptake of oxygen by nerve at rest and during activity.

METHOD

The common peroneal nerve, together with a portion of the sciatic nerve, was removed from 180-g female albino rats. The piece of nerve used was commonly 5 cm long and both ends of it were ligated with silk thread before it was removed from the animal. During the dissection a stream of CO_2 was allowed to flow over the dissection site to prevent the CO_2 tension in the nerve from becoming too low (MONNIER, 1952). The excised nerve, kept moist and under a stream of CO_2, was carefully freed of any adherent blood or connective tissue. The external sheath was not removed. The nerve was then placed in a thermostat in a vessel of Ringer's solution into which a gas mixture of 5% CO_2, 45% O_2, and 50% N_2 was bubbled. This washing served to remove minor traces of blood and to bring the nerve into thermal equilibrium at the temperature at which the measurement was to be made. The Ringer's solution used for this washing and also used in the flow respirometer was made with twice-distilled glass-condensed water and Merck's "NaCl for Biological Purposes." It had the following composition:

NaCl	136·0 mM
KCl	5·6 mM
$CaCl_2$	2·2 mM
Glucose	5·5 mM
$MgCl_2$	1·2 mM
$NaHCO_3$	16·2 mM

pH 7·34 when in equilibrium with 5% CO_2 at 30°.

After being washed, the nerve was placed in a flow respirometer of the type previously described in detail with respect to design and performance (CARLSON, BRINK, and BRONK, 1950). Briefly, such a respirometer consists of a well containing Ringer's solution and a glass capillary of diameter 1·5 mm

* Present address: Department of Physiology, State University of New York, College of Medicine at New York City, 450 Clarkson Avenue Brooklyn 3, N.Y. This work was carried out during the tenure of a National Research Council-Rockefeller Foundation Post-Doctoral Fellowship in the Medical Sciences.

† Present address: The Rockefeller Institute for Medical Research, New York, N.Y. This work was aided by grants to D. W. BRONK from the Supreme Council Scottish Rite Masons.

through which the solution is withdrawn from the well at a slow constant rate (in these experiments about 0·5 c.c. per hour). Gas of known CO_2 and O_2 content is continuously bubbled into the solution in the well. All gases used contained 5% CO_2 by volume unless otherwise specifically stated. The oxygen concentration in the capillary, about 3 cm from the well, is measured by electrolytic reduction with an oxygen cathode. Knowledge of the flow-rate, oxygen solubility, and the oxygen concentrations in the well and at the end of the capillary permits the calculation of the rate of oxygen uptake of a nerve placed in the capillary. The electrode is calibrated by observing the current flow during polarization of the oxygen cathode at various oxygen concentrations. Stimulating electrodes are mounted in the well above the level of the Ringer's solution, and recording electrodes are mounted across the capillary. The respirometer is contained in a thermostatically controlled chamber.

The sciatic portion of the nerve was placed on the stimulating electrodes and the common peroneal branch was drawn into the capillary of the respirometer. The common peroneal upon which the actual measurement was made is about 0·5 mm in diameter and about 23 mm long. Oxygen uptake of nerve not conducting impulses was determined by observing the empty-chamber oxygen concentration, inserting the nerve, waiting only until the new level of oxygen concentration at the electrode had been steady for 30 min, and then removing the nerve to be sure that the oxygen cathode current returned to the original empty-chamber value. This method served to detect the occasional experiment in which introduction of the nerve had altered the sensitivity of the oxygen cathode. By this method the rate of oxygen uptake may be determined with an overall precision of about 5%. The alteration of oxygen cathode sensitivity by introduction of the nerve was rather frequent until the procedure of washing the nerve in Ringer's solution for a few minutes was adopted. The sensitivity of the oxygen cathode may be unaltered by the first introduction of the nerve, but it may be reduced as much as 10%. Subsequent reinsertion and removal of the nerve does not have a further effect on the sensitivity.

Having thus obtained a value for the increment of electrode current corresponding to the oxygen uptake of the nerve, calibration of the electrode was made by shifting the gas bubbling in the well to gas of a different oxygen content. After a return to the original gas, the nerve was reinserted and could be stimulated to determine the effect of activity upon its rate of oxygen uptake (Fig. 1).

VOL
1
1956/

RESULTS

Resting metabolism. It proved difficult to obtain reliable and reproducible values for the resting rate of oxygen uptake at high temperatures. In particular, at any temperature above 35° the apparent resting uptake was constant for only 2 hours, and then tended to increase steadily with time, doubling after about 8 hours. The conditions of high-oxygen concentration, high temperature, and the presence of glucose and HCO_3^- are favourable to the growth of oxygen-consuming micro-organisms, and the presence of such micro-organisms undoubtedly contributed to a change in the properties of the nerve. The oxygen uptake of frog's nerve at rest had been shown to increase with time in a similar way, but much more slowly (BRINK *et al.*, 1952). Because of this increase with time, present to some extent at all temperatures, all the values given for resting uptake (Table 1) were obtained within 2 hours after excision, that is, before the increase had begun.

The most convenient temperature at which to study the oxygen uptake of this nerve was 30°. At that temperature the tendency of the resting respiration to drift with time was much less than at 35°. On the other hand at temperatures lower than 30° the ability of the nerve to conduct 200 impulses per sec for long times is impaired. It is probable that the temperature of the common peroneal nerve *in situ* is much closer to 30° than to the temperature of the body cavity of the rat. The common peroneal is near the surface in the thin hind limb, which has a high ratio of surface area to volume. Measurements of temperature by insertion of thermocouples under the skin of the hind leg confirmed the view that the temperature there is as much as 5° below the temperature of the body cavity.

Experiments were done on two nerves at 30° to test the effect of oxygen concentration on resting uptake. The resting uptake was found to be independent of oxygen concentration over a range of concentrations of O_2 in the gas bubbling in the well from 30% to 95%. The same result was found in many other experiments at 25°, 30°, and 35° in which resting respiration was determined at two different oxygen concentrations, either 30% and 45% or 45% and 60%. In general, such experiments were made difficult by the change in rate of uptake with time.

TABLE 1.—THE RATE OF OXYGEN UPTAKE OF THE PERIPHERAL NERVE OF RAT AT REST AND THE INCREASE IN RATE OF UPTAKE WITH ACTIVITY, BOTH AS A FUNCTION OF TEMPERATURE

The rates are given as mm^3 oxygen per gram wet weight of nerve per hour. The figures in the brackets give the range of observed values and the number of values included in each average.

	25°C	30°C	35°C
Resting	134 (114–156; 4)	200 (182–220; 6)	323 (262–409; 5)
50/sec	40·1 (33–47; 4)	37 (35·5–38·3; 5)	39·5 (37–41·5; 4)
100/sec	59·3 (50–69; 3)	(65–69; 4)	66·8 (57–77; 3)
200/sec	—	82 (64–89; 5)	79 (65–89; 3)

The resting uptake at 25°, 30°, and 35° is shown in Table 1 and Fig. 2. The Q_{10} of the resting rate of oxygen uptake by nerve at rest is 2·4 over the range 25°–35°.

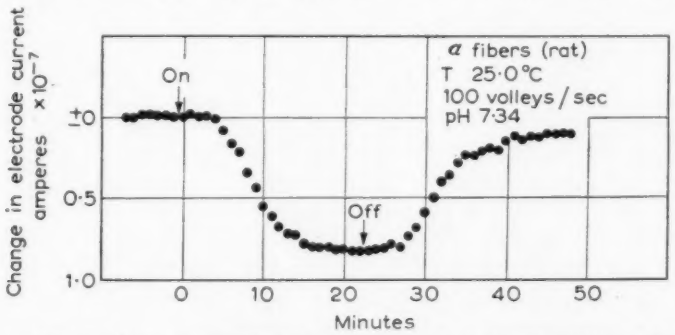


FIG. 1.—Change in current at the oxygen cathode during stimulation of the nerve. Values are taken from an actual experiment.

Rate of oxygen uptake by active nerve. The determination of the change in steady-state rate of oxygen uptake caused by activity in the nerve is somewhat less difficult than the determination of resting uptake. This results from the possibility of comparing the difference in the resting and active steady-state rates, using a calibration obtained by changing gas concentration while the nerve is in the capillary. The problem of alteration of electrode sensitivity secondary to insertion and removal of the nerve therefore does not arise. Furthermore, the activity increments do not change by more than 5% over the course of 12 hours. On the other hand, the increment of

oxygen uptake with stimulation at, say, 50 per sec at 35°, is such a small percentage of the resting uptake that it may be detected only by balancing out much of the signal and greatly amplifying the remainder (CARLSON, BRINK, and BRONK, 1950). Apparent drift in the baseline of resting uptake then becomes amplified in the same degree.

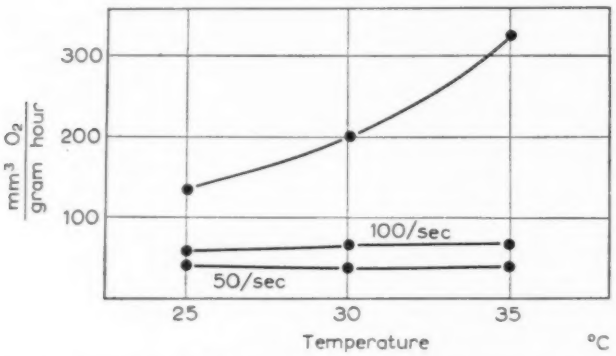


FIG. 2.—The oxygen uptake in mm^3/g hour of peripheral nerve of rat, as a function of temperature. Upper curve: oxygen uptake of the nerve not conducting impulses. Lower curves: Increment in oxygen uptake resulting from the conduction of impulses by the *A*-α fibres at the rate of 50/sec and 100/sec.

The *A*-alpha fibres were stimulated in the experiments designed to measure activity respiration. These fibres were stimulated maximally and the compound action potential was observed throughout each period of stimulation. When a nerve is in a steady state, as indicated by a steady rate of oxygen uptake, and is made to conduct 50, 100, or 200 impulses per second, the oxygen consumption rises rapidly (within minutes) to a new steady rate, as shown in Fig. 1. The magnitude of the new steady rate depends upon the frequency of stimulation. The nerve will maintain this new steady rate for at least 30 min unless conduction is failing (as indicated by the reduced area of the compound action potential), in which case the rate of uptake will drift back toward the resting level. There is no evidence that a properly conducting nerve cannot hold a steady rate of oxygen uptake for longer than 30 min, but it is difficult to show that it can, because of the drift in the baseline of resting uptake.

The values for the difference between the steady rates of oxygen uptake at rest and during activity are given in Table 1 and Fig. 2. In the experiments shown, the nerves all reached a new steady rate of uptake when stimulated and returned to the original resting rate of uptake upon cessation of stimulation. No value is given for the increment corresponding to 200 impulses per second at 25°, because at 25° the nerves would not conduct for long at that frequency.

The most remarkable property of the activity increments is their apparent temperature independence previously reported in the discussion following BRINK *et al.* (1952). Such a difference in the temperature dependence of the rate of uptake at rest and the increments of uptake with activity may be considered as evidence for some difference in the enzymic processes whose rates are measured by these two components of oxygen uptake. A similar observation of relative temperature insensitivity of the activity respiration increment in frog nerve has been made recently.

VOL
1
1956/

DISCUSSION

The above results indicate that the peripheral nerve of rat is not profoundly different from that of frog in its oxygen-uptake characteristics. When stimulated, its metabolic processes change to a new steady state, as indicated by a higher steady rate of oxygen uptake which is maintained until stimulation is stopped. When stimulation is stopped, the rate of oxygen uptake returns to the value characteristic of resting nerve. The rate of oxygen uptake of resting nerve of either species has a Q_{10} between 2 and 3, whereas the increments of oxygen uptake associated with activity are less sensitive to temperature. The increments of uptake associated with activity in rat nerve tend to saturate somewhat above 200/sec rather than around 100/sec, as with frog nerve. There is one notable difference not previously referred to: the approach to, and return from, the higher steady levels of oxygen uptake associated with activity occur much more swiftly than do the comparable changes seen in frog nerve. This fact was not discussed in the results because no precise estimate of the time-constants can be given. All that can be said is that the changes occurring in the nerve are too rapid to be resolved by the method used. The time-constants found in frog nerve are on the other hand so large that the transients arising in the reactions of the nerve last much longer than those inherent in the flow respirometer.

In general, it may be said that the above results establish the absolute values of oxygen uptake at rest and during the conduction of impulses for one type of mammalian nerve, and suggest that the processes determining the relation of oxygen metabolism to conduction of impulses in rat nerve are similar to those determining this relation in frog nerve.

SUMMARY

1. The resting rate of uptake of oxygen by rat peripheral nerve has been determined. The temperature coefficient of this resting rate has been determined.

2. Rat peripheral nerve has been shown to reach a new steady-state rate of oxygen uptake when stimulated, and this rate has been determined at various temperatures and frequencies.

3. Increases in oxygen uptake with activity have been found to have a temperature dependence entirely different from that of the resting uptake.

REFERENCES

BRINK F., CARLSON F. D., and CONNELLY C. M. (1952) *Cold Spring Harbor Symposium on Quantitative Biology* **17,** 53–67.

CARLSON F. D., BRINK F. JR., and BRONK D. W. (1950) *Rev. Scientific Instrum.* **21,** 923–932.

MONNIER A. M. (1952) *Cold Spring Harbor Symposium on Quantatitive Biology* **17,** 69–95.

Journal of Neurochemistry, 1957, Vol. 1, pp. 250-255. Pergamon Press Ltd., London

AMMONIA FORMATION IN BRAIN—III

THE ROLE OF THE PROTEIN AMIDE GROUPS AND OF HEXOSAMINES

H. WEIL-MALHERBE and A. C. DRYSDALE
Research Department, Runwell Hospital, Wickford, Essex

(*Received* 10 *December* 1956)

VOL
1
1956/

THE origin and mechanism of the ammonia formation occurring in brain slices during their incubation in a glucose-free saline have been discussed in an earlier publication (No. 1 of this series, WEIL-MALHERBE and GREEN, 1955). The conclusion was reached that none of five deaminating brain enzymes was likely to be involved; it was suggested that the formation of ammonia in brain slices was intimately linked with proteolysis, because ammonia and nonprotein nitrogen were increased under similar conditions.

Recent work from other laboratories has suggested possibilities which we had not considered. Thus VRBA (1955a) found that the level of protein-bound amide groups in rat brain was lowered after physical exhaustion and that, at the same time, there was an increase of free glutamine and a decrease of free glutamic acid, indicating an increased liberation of ammonia (VRBA, 1955b; 1956). The protein-amide fraction was therefore examined as a potential source of the ammonia formed by brain slices.

A sixth brain enzyme has now to be added to the deaminases listed by WEIL-MALHERBE and GREEN (1955). It liberates ammonia from glucosamine 6-phosphate (FAULKNER and QUASTEL, 1956; LELOIR and CARDINI, 1956; NAKADA and WOLFE, 1956). Hexosamines are known to be constituents of brain gangliosides (BLIX, SVENNERHOLM, and WERNER, 1950; KLENK, 1951; BRANTE, 1957). Their presence in the interstitial "ground substance" of brain has been demonstrated histochemically (FREEDMAN, 1953; HESS, 1953).

A major part of the ammonia formation in brain slices depends on the integrity of the electron transport and oxidative phosphorylation systems (WEIL-MALHERBE and GREEN, 1955). If it could be shown that hexosamines are the precursors of ammonia, this requirement could be explained, since hexosamines are only deaminated after their phosphorylation by adenosine triphosphate. It has already been shown in 1941 by LUTWAK-MANN that hexosamines added *in vitro* are deaminated by brain slices and that this reaction only occurs aerobically. The concentrations of acid-insoluble and total hexosamines were therefore studied in brain slices after various periods of incubation.

METHODS

The preparation and incubation of brain slices, the correction of the weights of slices for swelling, the preparation of protein-free filtrates, and the estimation of ammonia in Conway units were carried out as previously described (WEIL-MALHERBE and GREEN, 1955). About 100 mg of brain slices (corrected initial wet weight) were used per sample.

Estimation of protein amide nitrogen

The tissue insoluble in 5% trichloroacetic acid was extracted with lipid solvents according to LESLIE and DAVIDSON (1951). The dry residue was heated with 0·2 ml 98% (w/v) formic acid in a boiling-water bath for 5 min (GURNANI, KUMTA, and SAHASRABUDHE, 1955) and then refluxed for 2 hr with 2 ml 2 N-HCl. After cooling, the solution was made up to 6 ml with water, and filtered; 0·5-ml portions of the filtrate were used for the estimation of ammonia in Conway units.

Treatment of the tissue with hot formic acid greatly reduced the concentration of HCl and the time required for complete hydrolysis of the protein-amide nitrogen. No further formation of ammonia was found when the hydrolysis was allowed to proceed for more than 2 hr. Under these relatively mild conditions no ammonia was liberated from serine or threonine.

Estimation of hexosamines

(a) *Acid-insoluble hexosamines.* The tissue insoluble in 5% trichloroacetic acid was suspended in 2 ml 2 N-HCl and sealed in a small test-tube. It was then heated in a boiling-water bath for 16 hr. The hydrolysate was freed from HCl by filtration through a column of 5 g Dowex-2 (8% cross-linked, 50–100 mesh, OH^--form; diameter of column about 6 mm). The column was washed with 23 ml water. The filtrate and washings were now passed over a column of 1 g Dowex-50 (10% cross-linked, 50–100 mesh, H^+-form); this retains cationic hexosamines but not neutral sugars, which interfere with the colorimetric analysis (BOAS, 1953). The resin column was washed with 10 ml water and the filtrate and washings were rejected. Elution was carried out with 4 ml 2 N-HCl, followed by 1 ml water. The eluate was neutralized with N-NaOH and made up to 14 ml. For the colorimetric estimation (in duplicate) the method of RONDLE and MORGAN (1955) was adapted by increasing the scale threefold, i.e. 6 ml of the eluate were used in a final volume of 30 ml. The absorption was measured in a cell with a light-path of 4 cm.

(b) *Total hexosamines.* The tissue insoluble in trichloroacetic acid was hydrolysed as described above and the hydrolysate combined with the trichloroacetic acid filtrate. The solution was then filtered through Dowex-2. To cope with the increased amount of acid, a column of 7 g was used. Acid-soluble hexosamines, but not acid-soluble acetylhexosamines, are included in the estimate.

RESULTS

The present observations on the formation of ammonia by brain slices confirm the earlier ones (WEIL-MALHERBE and GREEN, 1955). They show that rat and guinea-pig brain slices are about equal in activity, that 2:4-dinitrophenol (DNP) is inhibitory after an initial lag-period, and that the absence of oxygen inhibits even more promptly and strongly (Table 1).

Estimations of the protein amide-nitrogen concentration in brain slices showed considerable scatter, not only in different animals, but also in slices from the same animal, suggesting that this fraction is not uniformly distributed in different parts of grey matter. A slight decrease was observed during the aerobic incubation of brain slices, but the differences are not significant (Table 1). Even if these differences were shown to be real, they would only account for about one-half of the ammonia evolved.

Although the ammonia formation in guinea-pig brain slices was strongly depressed by DNP and by anaerobic incubation, decreases of protein amide-nitrogen were found under these conditions which were somewhat larger than those observed when ammonia formation was not inhibited. The decrease due to DNP was significant in several samples.

The last column of Table 1 gives the sum of the two fractions. This should be constant if the protein amide-nitrogen were the sole precursor of ammonia. The differences observed were not significant, but the tendency is for an increase in the aerobic experiments, indicating the formation of ammonia from other sources, and for a decrease in the anaerobic experiments, indicating the disappearance of protein

TABLE 1.—CONCENTRATION OF AMMONIA AND PROTEIN AMIDE-NITROGEN DURING THE INCUBATION OF BRAIN SLICES IN GLUCOSE-FREE KREBS-BICARBONATE SALINE

Mean values ± standard error of mean (in μmoles/100 mg wet weight)

Time (hr)	Ammonia	Protein amide-N	Ammonia + protein amide-N
(a) *Guinea-pig brain, aerobic, 9 experiments*			
0	0·75 ± 0·109	7·88 ± 0·338	8·63 ± 0·418
0·5	*1·17 ± 0·166*	7·96 ± 0·372	9·13 ± 0·482
1	**1·71 ± 0·177**	7·71 ± 0·293	9·41 ± 0·453
2	**2·14 ± 0·212**	7·65 ± 0·373	9·80 ± 0·500
3	**2·52 ± 0·256**	7·65 ± 0·408	10·10 ± 0·640
5	**2·79 ± 0·248**	7·05 ± 0·405	9·82 ± 0·539
(b) *Rat brain, aerobic, 4 experiments*			
0	0·94 ± 0·086	8·23 ± 1·13	9·15 ± 1·07
0·5	*1·39 ± 0·132*	8·30 ± 0·217	9·70 ± 0·204
1	**1·93 ± 0·181**	7·01 ± 1·026	8·92 ± 0·942
2	**2·22 ± 0·135**	7·05 ± 1·24	9·27 ± 1·21
3	**2·49 ± 0·191**	7·25 ± 0·563	9·74 ± 0·451
5	**2·74 ± 0·164**	6·74 ± 0·394	9·48 ± 0·404
(c) *Guinea-pig brain, with* 10^{-3} M *2:4-dinitrophenol, 3 experiments*			
0	0·76 ± 0·090	8·83 ± 0·357	9·58 ± 0·280
0·5	1·06 ± 0·105	8·23 ± 0·336	9·29 ± 0·371
1	**1·63 ± 0·125**	**6·60 ± 0·109**	*8·23 ± 0·234*
2	**1·89 ± 0·065**	*6·64 ± 0·466*	8·53 ± 0·513
3	**1·69 ± 0·034**	*6·76 ± 0·546*	8·46 ± 0·552
5	**1·70 ± 0·171**	*6·53 ± 0·680*	8·24 ± 0·741
(d) *Guinea-pig brain, anaerobic, 4 experiments*			
0	0·676 ± 0·091	7·60 ± 0·706	8·26 ± 0·680
0·5	0·976 ± 0·171	7·24 ± 1·04	8·20 ± 1·160
1	0·930 ± 0·098	6·82 ± 0·807	7·75 ± 0·871
2	*1·230 ± 0·133*	6·85 ± 0·685	8·09 ± 0·803
3	*1·150 ± 0·173*	5·83 ± 0·821	6·97 ± 0·960
5	1·148 ± 0·275	6·06 ± 0·700	7·21 ± 0·966

Values which are significantly different from the initial value at the 5% level are shown in *italics*, those significantly different at the 1% level are shown in **bold type.**

amide-nitrogen without the appearance of ammonia. One of the decreases observed in the presence of DNP is significant at the 5% level.

The significance of these results was further tested by expressing, in each experiment, the values obtained on incubation as percentages of the initial value. In some cases all results obtained after incubation for more than a certain period were combined. Table 2 shows those results where the deviation from the initial value of 100 is significant.

Several trends, such as the decrease of the protein amide-nitrogen of rat brain slices after aerobic incubation for 1 hr or more (all results combined), thus appeared to be significant. The sum of ammonia and protein amide-nitrogen, on the other hand, did not change in this experiment. While these results are consistent with the formation of ammonia from protein amide groups, the experiments with guinea-pig brain slices had the opposite result: the protein amide fraction did not change, but

VOL 1 1956/

TABLE 2.—PROTEIN AMIDE-NITROGEN AND SUM OF AMMONIA AND PROTEIN AMIDE-NITROGEN AFTER INCUBATION OF BRAIN SLICES

Results were expressed as percentages of initial values (= 100) and the means and standard errors calculated. Only those figures which differ significantly from 100 are shown

Type of experiment	Time of incubation	Protein amide-N	Ammonia + protein amide-N
Guinea-pig, aerobic	1–5 hr (all samples)		**114 ± 3·85**
Rat, aerobic	1–5 hr (all samples)	**86·9 ± 3·68**	
Guinea-pig, with DNP	1 hr	**75·0 ± 4·36**	86·2 ± 5·02
	2 hr	75·7 ± 7·17	
	3 hr	**76·7 ± 3·66**	88·0 ± 3·25
	5 hr	74·8 ± 10·86	
	1–5 hr (all samples)	**75·5 ± 3·04**	**87·4 ± 2·97**
Guinea-pig, anaerobic	3–5 hr (last 2 samples)	**75·3 ± 4·92**	83·0 ± 5·61

Values shown in **bold type** are significant at 1% level, others at 5% level.

the sum of ammonia and protein amide-nitrogen significantly increased, presumably indicating the formation of ammonia from a different source.

A significant decrease of the protein amide fraction was found when guinea-pig brain slices were incubated in the presence of DNP for 1 hour or longer or when they were incubated anaerobically for 3 hr or more. Under the same conditions, the sum of ammonia and protein amide-nitrogen decreased significantly, indicating the disappearance of protein amide groups without a commensurate formation of ammonia.

The hexosamine content of brain slices after different times of incubation is shown in Table 3. Only the acid-insoluble fraction was analysed in the first two experiments; however, when the total and the acid-insoluble fractions of guinea-pig brain slices

TABLE 3.—CONCENTRATION OF HEXOSAMINES DURING THE INCUBATION OF BRAIN SLICES IN KREBS-BICARBONATE SALINE (GLUCOSE-FREE, EXCEPT LAST SERIES)

Mean values, if more than one experiment (μmoles/100 mg wet weight)

Species	Type of experiment	Fraction analysed	No. of experiments	Hexosamines after				
				0	1	2	3	5 hr
Guinea-pig	aerobic	acid-insoluble	3	0·551	0·524	0·572	0·526	0·556
			3	range 0·488–0·647	0·508–0·544	0·519–0·620	0·472–0·553	0·539–0·579
Guinea-pig	anaerobic	acid-insoluble		0·514	0·512	0·493	0·540	0·518
				range 0·466–0·586	0·463–0·564	0·452–0·525	0·516–0·564	0·446–0·592
Guinea-pig	aerobic	total	1	0·402	0·363	0·444	0·430	0·485
			2	0·573	0·558	0·570	0·632	0·571
Rat	aerobic	total						
Rat	aerobic with 0·2% glucose	total	3	0·666	0·685	0·650	0·666	0·595
				range 0·636–0·697	0·585–0·765	0·620–0·683	0·647–0·705	0·570–0·620

are compared, it appears that the slices contained very little, if any, acid-soluble hexosamine. The figures for total hexosamine were slightly higher with slices of rat brain than with those of guinea-pig brain.

The results show that the concentration of hexosamine remained constant throughout the incubation.

In one series of experiments the effect of glucose on the concentration of hexosamine in rat brain slices was studied, but no net synthesis occurred.

DISCUSSION

On the basis of the foregoing results, the possibility of hexosamines acting as the major precursor of ammonia in brain slices may be dismissed: not only is the concentration of hexosamines insufficient to provide for more than a third to a fifth of the ammonia actually formed, but what little there is remains constant during the incubation.

The position is less clear-cut as regards the protein-amide fraction. During the aerobic incubation of guinea-pig brain slices there was a slight, but probably significant, increase of the sum of ammonia and protein amide-nitrogen; some at least of the ammonia therefore probably came from sources other than protein amide groups. On the other hand, the mere fact that the disappearance of protein amide groups tallies with the formation of ammonia does not in itself justify the conclusion of a causal relationship, unless it can be shown that hydrolysis is the only metabolic reaction of which protein amide groups are capable. Actually, significant decreases of the sum of the two fractions were found when brain slices were incubated with DNP or in nitrogen, suggesting that under these conditions protein amide groups disappeared by paths other than the formation of ammonia.

Our figures for the concentration of protein amide groups in slices of grey matter of brain are a little lower than those found by VRBA (1955a) in whole brains frozen *in vivo*. This may be due to differences in animals used, to differences of technique, or finally to a rapid post-mortem hydrolysis of a particularly labile fraction. If such a fraction exists, it may account for the observations of VRBA.

It would be easier to accept the hypothesis of ammonia formation from protein amide groups, if an enzyme were known to bring about this reaction. However, it is generally assumed that the formation of ammonia which occurs during the action of proteolytic enzymes is due to the spontaneous hydrolysis of glutamine and asparagine or their simple peptides (MELVILLE, 1935; TERROINE and LAURESCO, 1935). As far as the present writers are aware, amidases which attack proteins have not yet been described.

The conclusion to be drawn from this study therefore is that hexosamines do not contribute to the ammonia formation of brain slices and that protein amide groups, if they contribute at all, do not account for all of the ammonia formed. The problem of its origin still awaits clarification.

SUMMARY

The possibility of protein amide groups or hexosamines acting as the precursors of the ammonia formed during the incubation of brain slices in a glucose-free medium was investigated, and the following observations were made:

(1) During the aerobic incubation of rat brain slices the concentration of protein

VOL
1
1956/

amide groups decreased slightly and the sum of ammonia and protein amide nitrogen remained constant. While these findings are compatible with the precursor function of protein amide groups, the latter did not change during the incubation of guinea-pig brain slices and the sum (ammonia + protein amide nitrogen) increased, indicating the formation of ammonia from other sources.

(2) When ammonia formation was strongly inhibited, either by the addition of 2:4-dinitrophenol or by the exclusion of oxygen, protein amide groups and the sum (ammonia + protein amide nitrogen) both decreased, suggesting a metabolism of protein amide groups other than by hydrolysis to free ammonia.

(3) The concentration of hexosamines in brain slices did not change during aerobic or anaerobic incubation. In any case, it is too small to account for more than a fraction of the ammonia formed.

REFERENCES

Blix G., Svennerholm L. and Werner J. (1950) *Acta chem. scand.* **4,** 717.
Boas N. F. (1953) *J. biol. Chem.* **204,** 553.
Brante G. (1957) *Metabolism of the Nervous System* (Ed. D. Richter) Pergamon Press, London.
Faulkner P. and Quastel J. H. (1956) *Nature* **177,** 1216.
Freedman B. (1953) *Anat. Rec.* **115,** 265.
Gurnani S. U., Kumta U. S. and Sahasrabudhe M. B. (1955) *Biochim. biophys. Acta* **16,** 553.
Hess A. (1953) *J. comp. Neurol.* **98,** 69.
Klenk E. (1951) *Z. Physiol. Chem.* **288,** 216.
Leloir L. F. and Cardini C. E. (1956) *Biochim. biophys. Acta* **20,** 33.
Leslie I. and Davidson J. N. (1951) *Biochim. biophys. Acta* **7,** 413.
Lutwak-Mann C. (1941) *Biochem. J.* **35,** 610.
Melville J. (1935) *Biochem. J.* **29,** 179.
Nakada H. I. and Wolfe J. B. (1956) *Fed. Proc.* **15,** 135.
Rondle C. J. M. and Morgan W. T. J. (1955) *Biochem. J.* **61,** 586.
Terroine E. F. and Lauresco C. (1935) *Arch. Internat. Physiol.* **42,** 205.
Vrba R. (1955a) *Nature* **176,** 117.
Vrba R. (1955b) *Nature* **176,** 1258.
Vrba R. (1956) *J. Neurochem.* **1,** 12.
Weil-Malherbe H. and Green R. H. (1955) *Biochem. J.* **61,** 210.

Journal of Neurochemistry, 1957, Vol. 1, pp. 256-259. Pergamon Press Ltd., London

STRUCTURAL AND CHEMICAL DIFFERENCES BETWEEN OPTIC AND SCIATIC NERVE MYELINS

J. B. FINEAN, J. N. HAWTHORNE, and J. D. E. PATTERSON
Department of Medical Biochemistry and Pharmacology, University of Birmingham

(*Received* 10 *December* 1956)

A COMPARISON of X-ray diffraction data from sciatic nerve, optic nerve, brain white matter, and spinal cord (FINEAN, 1955) has indicated significant differences in myelin structure in peripheral nerve and central nervous system. In the case of fresh myelin these differences are in the size of the structural repeating unit in the myelin sheath, and in the distribution of diffracting material within this unit. The differences are emphasized by the observation that the peripheral nerve myelin appears to undergo irreversible changes in structure under conditions which produce only reversible changes in the myelin of central origin. On the other hand, similarities are indicated from the effects of osmium tetroxide and of mercuric chloride on the diffraction patterns of the two systems (FINEAN, 1957). The structural arrangement suggested for the peripheral nerve myelin on the basis of X-ray diffraction data features two lipoprotein layers (FINEAN, 1953). These are very similar in general structural arrangement but differ by some factor which has an appreciable scattering power for X rays. However, the diffraction data indicates that this factor can be removed, or distributed evenly between the two layers, by relatively mild treatment. The structural unit of myelin of central origin may again include two very similar layers, but the "difference factor" can have only a low scattering power for X rays. Furthermore, if these are again lipoprotein layers, then they are less easily modified than those of peripheral nerve myelin.

Electron micrographs have so far shown no significant differences in type of layering in the two systems. As would be expected from the X-ray diffraction data (FINEAN, 1954), the deposition of osmium in the layered structure emphasizes the difference factor between the two layers in the fundamental structural repeating unit. There is a very heavy deposition over a distance of about 30 Å at the full period and only a relatively light deposition at the half-period (SJÖSTRAND, 1953). However, in the central nervous system a complete halving of the fundamental period is occasionally found (FERNÁNDEZ-MORÁN, 1957), an occurrence which may have some significance in relation to the predicted similarities of the two layers in the fundamental unit. The important question of the sites of osmium deposition in lipoprotein systems can be considered in general terms, the probability being that the lightest deposition of osmium occurs in the region of the lipid hydrocarbon chains and the heaviest at ionic lipoprotein or lipid-lipid interfaces. The heavy band of deposition in the myelin layers probably covers two lipoprotein interfaces on either side of a thin protein layer. The lighter intraperiod deposit may also be at a lipoprotein interface which would either have to differ somewhat in composition from the first one or be less

VOL
1
1956/

accessible to osmium tetroxide. The idea of a lipoprotein interface again at the half-period rather than a lipid-lipid interface is preferred because of the X-ray diffraction evidence for the possibility of producing complete "halving" of the fundamental unit by relatively simple procedures (FINEAN, 1953).

Previous data on the chemical composition of various types of nerve tissue do not provide any detailed comparison of optic and sciatic material, and comparison of brain white matter and peripheral nerve have seldom, if ever, been carried out under carefully controlled conditions. However, one significant point of contrast relating to brain white matter and sciatic nerve is the observation that the white matter contains about twice as much inositide phosphorus as the sciatic nerve (LOGAN *et al.*, 1952). The inositide phosphorus is described as that part of the protein-bound phosphorus which is not accounted for as phosphoprotein or nucleic acid.

The proteolipids, first isolated from brain white matter by FOLCH and LEES (1951), are of great interest in relation to myelin structure. Similar fractions have been isolated from bovine optic nerve (BAKER, 1957), and the possibility that the protein in these fractions is taken into solution in chloroform-methanol by virtue of strongly associated lipid components could be of great significance in relation to the types of linkages prominent in the lipoprotein association in the myelin. In seeking a chemical basis for the observed structural differences between myelin in the central nervous system and that of peripheral nerve, particular emphasis has been laid in the first place on proteolipid components. Optic and sciatic nerves were chosen as the most suitable materials for combined structural and chemical studies, and this paper reports preliminary results of the chemical comparison.

MATERIALS AND METHODS

The main experiments were carried out on material from the horse, and a number of similar experiments were made with cow and with rabbit material. In all cases, comparable wet weights of optic and sciatic nerves were selected from the same animal within an hour or so of killing it, and extracted by homogenizing in 2:1 chloroform methanol (40 ml/g wet weight). After filtering, the extracts were equilibrated against 20 vols of water, following the procedure described by FOLCH *et al.* In some cases the "fluff" separating at the chloroform-water interface was isolated by freezing and filtering after removal of the water layer. In others the water was removed and the fluff redissolved by adding methanol to the chloroform layer. The "proteolipid protein" was separated by adding 5% water to the proteolipid solutions and evaporating to dryness. This was repeated twice; the water was finally removed by introducing ethanol and then evaporating. The lipid was redissolved in chloroform, leaving an insoluble material behind. This material was identified as a protein by chromatography of the amino acids released by acid hydrolysis, and it was analysed for nitrogen (micro-Kjeldahl), phosphorus (KING, 1932) and inositol (BÖHM and RICHARZ, 1954). The chloroform-soluble lipids were analysed for phosphorus, and a chromatographic study was made of the phosphatide bases liberated by acid (5 N-HCl) hydrolysis. An estimation of the relative amounts of choline, ethanolamine, and serine was made from a consideration of areas of spots on the chromatogram (FISHER *et al.*, 1948).

RESULTS

The observations on the horse, cow, and rabbit materials were essentially the same. Quantitative results are derived from five experiments with horse nerves.

On carrying out the Folch fractionation, similar amounts of interfacial fluff were obtained from both sciatic and optic nerves. However, whereas the sciatic nerve fluff readily dissolved in chloroform to which 12% methanol had been added and was subsequently shown to contain very little protein, the optic nerve fluff was far less

readily dissolved and contained appreciable amounts of protein. In the case of the $CHCl_3$-soluble material separated from the fluff, that from optic was again found to contain appreciable amounts of protein, but the sciatic gave negligible protein. Comparing the total proteolipid proteins (fluff plus chloroform layer), it was found that the sciatic yielded almost negligible quantities as compared with the optic. The quantitative figures are given in Table 1. They are related to wet weight and also to the total phosphorus content of the extract.

TABLE 1

	% Proteolipid protein related to wet weight	% Phosphorus related to wet weight	Ratio (wt/wt) protein/phosphorus	Ratio optic/sciatic proteolipid protein weights	
				Related to wet weight	Related to total phosphorus
Optic I *			7·8		17·6
Sciatic I	0·056	0·12	0·45		
Optic II	1·6	0·21	7·5	27·5	17·2
Sciatic II	0·063	0·14	0·44		
Optic III	1·55	0·20	7·7	20·5	16·1
Sciatic III	0·076	0·15	0·48		
Optic IV *	1·4			21·5	
Sciatic IV	0·064	0·12	0·51		
Optic V	1·6	0·22	7·2	29·0	16·5
Sciatic V	0·54	0·12	0·43		

* Experiment I. Gross error in wet weight of optic nerve.
Experiment IV. Gross error in estimation of phosphorus content of optic nerve.

The proteolipid proteins derived from the two systems showed similar amino-acid compositions, as demonstrated by two-dimensional paper chromatography, but the optic nerve proteolipid protein contained approximately twice as much phosphorus (0·42% to 0·20%) and twice as much inositol (1·41% to 0·66%) as that from sciatic nerve. In both cases the inositol appeared to be present as a diphosphoinositide.

Comparison of phosphorus-containing end groups of the total lipid component by chromatography showed no significant differences between optic and sciatic material.

DISCUSSION

The most striking difference between optic and sciatic nerves revealed by these preliminary experiments is the almost complete absence of proteolipid protein in the case of the sciatic nerve. If the proteolipid is indeed a remnant of the myelin, then the implication would be that the lipoprotein association in sciatic nerve differs considerably, either in type or in strength, from that in the optic. The fact that the optic proteolipid protein contains considerably more phosphorus and inositol than that

VOL 1 1956/

from sciatic might mean that these factors are important in forming strong linkages between lipid and protein components of the two systems. Furthermore, as the lipid components are shown to be very much alike in composition, it may be that the structures depend on relatively few such linkages for their stability. However, the amount of protein that is extracted from the sciatic nerve is very small, and it would be useful to have information on the myelin protein left in the residue after extraction.

These initial experiments give a useful lead as to where the chemical factors which underlie the structural differences in optic and sciatic nerve myelins might lie, and the lead is being followed.

We are grateful to Professor A. C. FRAZER for his continued interest in this work.

REFERENCES

BAKER R. G. (1957) Unpublished results.

BÖHM P. and RICHARZ G. R. (1954) *Z. Physiol. Chem.* **298,** 110.

FERNÁNDEZ-MORÁN H. (1957) *Metabolism of the Nervous System* (Ed. D. RICHTER) Pergamon Press, London.

FINEAN J. B. (1953) *Exp. Cell. Res.* **5,** 202.

FINEAN J. B. (1954) *Exp. Cell. Res.* **6,** 283.

FINEAN J. B. (1955) *Internat. Colloquium on Biochemical Problems of Lipids, Brussels* p. 82.

FINEAN J. B. (1957) *Metabolism of the Nervous System* (Ed. D. RICHTER) Pergamon Press, London.

FISHER R. B., PARSON D. S., and MORRISON G. A. (1948) *Nature* **161,** 764.

FOLCH J. and LEES M. (1951) *J. biol. Chem.* **191,** 807.

KING E. J. (1932) *Biochem. J.* **26,** 292.

LOGAN J. E., MANELL W. A., and ROSSITER R. J. (1952) *Biochem. J.* **51,** 470.

SJÖSTRAND F. S. (1953) *Experientia* **9,** 68.

Journal of Neurochemistry, 1957, Vol. 1, pp. 260-271. Pergamon Press Ltd., London

CEREBROCUPREIN I. A COPPER-CONTAINING PROTEIN ISOLATED FROM BRAIN

HUNTINGTON PORTER and JORDI FOLCH

with the technical assistance of

JURIS BEGGROVS and SALLY AINSWORTH

The McLean Hospital Research Laboratory, the Neurological Service, Massachusetts General Hospital, and the Departments of Neurology and Biological Chemistry Harvard Medical School, Boston

(*Received* 28 *December* 1956)

VOL
1
1956/

PREVIOUS reports (PORTER and FOLCH, 1954; 1957) have described the separation from brain tissue under copper-free conditions of three different copper-containing fractions: fraction I, which is extracted from the tissue with 0·1 M-acetate buffer pH 4·5; fraction II, which is obtained by subsequent extraction of the tissue residue with water at pH 3·5 and vanishing ionic strength; and the residual fraction III. These fractionation techniques have also been applied to brains of patients with hepatolenticular degeneration (Wilson's disease), in which the total copper content was markedly increased (PORTER and FOLCH, 1957). The present paper describes two different procedures* for the preparation from fraction I of a protein sub-fraction containing 0·14% copper and the isolation from it, by preparative paper electrophoresis, of a green brain copper protein which has a copper content of 0·25 to 0·3% and which appears to be 85% homogeneous both by ultracentrifugation and by analytical paper electrophoresis. Since the isolated copper protein appears to differ from those previously described and since there appears to be more than one type of copper protein in brain, it is suggested that this protein be designated for descriptive purposes as cerebrocuprein I.

METHODS

The extensive special precautions to avoid contamination with extraneous copper which were taken throughout the fractionation procedures and the methods used for tissue homogenization and for copper analysis were identical with those employed in previous work (PORTER and FOLCH, 1957). The meninges were stripped from each brain under cold redistilled water. After mincing by shearing between two wooden tongue depressors, the tissue was homogenized by freezing at -10°, followed by partial thawing and grinding in a mortar with 90-mesh alundum. All glassware was cleaned by allowing it to stand overnight in 6 N-HCl prior to use, followed by exhaustive rinsing; the alundum used in grinding the tissue was similarly cleaned with the addition of an initial 2 hour period in contact with boiling 6 N-HCl. All water used was redistilled over glass. The acetate buffers, which when made from reagent-grade chemicals contained appreciable copper, were made copper-free by the addition to each litre of buffer of 1 ml of a 2% (w/v) solution of sodium diethyldithiocarbamate followed by extraction four times with 10 ml portions of carbon tetrachloride. In order to confirm that the conditions employed were not introducing extraneous copper, the grinding and tissue extraction procedure was carried out in quadruplicate as a blank without tissue but using alundum and acetate buffer both freed from extraneous copper as described above. It was demonstrated that the grinding and tissue extraction procedure would not contribute more than 0·07 μg copper per gram of

* One of these procedures, subsequently referred to as method A, has already been presented in a preliminary report (PORTER and FOLCH, 1956).

fresh tissue. That the copper in the selected fractions was, in fact, derived from brain and not from contamination was further established by the analysis of all the discarded fractions. It was thus possible to demonstrate at each step in the isolation procedure that the sum of the copper in the different fractions did not exceed the amount of copper present in the material from which the fractions had been obtained. Any preparation which failed to satisfy this condition was discarded *in toto*.

Unless otherwise stated, all fractionation procedures were carried out in cold rooms, either at 4° during manipulation of aqueous solutions, or at −10° or lower during manipulation of acetone-water mixtures. Acetone to be added to aqueous solutions was precooled to −10° or lower and the addition carried out gradually with constant stirring to avoid local overconcentration or rise in temperature. The stated acetone concentrations refer to volume per volume. Unless otherwise stated, adjustments of pH were made by the dropwise addition of 0·1 N-NaOH or HCl.

Paper electrophoresis was carried out in an apparatus of the modified horizontal type (LATNER, 1952). Copper-free sodium diethylbarbiturate buffer of pH 8·6 and ionic strength 0·1 was used in all electrophoresis experiments. Whatman No. 31 paper was used in the preparative runs and Whatman No. 1 paper in runs for protein analysis. Strips used for protein analysis were stained with bromphenol blue and their optical densities recorded.

Copper determinations were carried out in duplicate by the method of EDEN and GREEN (1940), modified as previously reported (PORTER, 1951). The total nitrogen of the isolated copper protein was estimated in triplicate by wet digestion with H_2SO_4, K_2SO_4, $CuSO_4$, and H_2O_2, followed by nesslerization of the digest (PETERS and VAN SLYKE, 1932). Absorption spectra of the isolated copper protein were determined in a Beckmann model DU spectrophotometer, using a micro curvette of 1 cm light path and approximately 0·3 ml capacity.

ISOLATION PROCEDURES

Fraction I was prepared as previously described (PORTER and FOLCH, 1957) by grinding the fresh tissue with 90-mesh alundum while freezing and thawing, followed by extraction with 0·1 M-sodium acetate buffer pH 4·5 (2 ml buffer per gram fresh tissue) and centrifugation. 4 to 5 kg of whole bovine brain were used as the starting material for each preparation. Only 6 to 8 litres of extract (fraction I) were obtained, because with the buffer-to-tissue ratio selected a relatively large proportion of buffer extract remained with the tissue residue. After allowance for this loss, fraction I accounted for over 40% of the total brain copper and less than 5% of the total brain proteins. To the extract was added acetone, precooled to 20°, to a concentration of 45% (v/v), and the mixture allowed to stand for 24 hr. The resulting precipitate was removed by filtration and discarded. Acetone was added to the filtrate to a final concentration of 75% acetone, and the mixture allowed to stand for 24 hr. By the end of this period, the newly formed precipitate had settled almost completely. After siphoning off the bulk of the clear supernatant, the precipitate was collected by centrifugation, washed successively with acetone and ether, and dried at −10°, first in air and then in a vacuum desiccator. The dried material contained about 0·01% copper and accounted for about 75% of the copper of fraction I. It will be referred to henceforth as the 0·01% copper fraction.

Two alternative procedures have been developed for the isolation from this material of a fraction containing 0·14% copper. The copper content of the successive copper-rich fractions obtained in the course of these isolation procedures is shown in Table 1. In calculating yields of copper and of protein per 100 g fresh tissue, the aliquots of copper-rich fractions utilized in pilot experiments or otherwise subtracted during the fractionation procedure have been taken into acccount.

Preparation of 0·14% copper-containing fractions

Method A: *Repeated acetone precipitation at low temperature, at* pH 5·2, *and at vanishing ionic strength.* Throughout this method, centrifugation was used to collect insoluble fractions. The 0·01% copper fraction was suspended in 40-fold its weight of water, the suspension was dialysed against several changes of redistilled water for 72 hr, and the material remaining insoluble was discarded. The supernatant solution was adjusted to pH 8·5 and a small amount of resulting precipitate was discarded. The solution was again dialysed and any newly formed precipitate was discarded. The pH of the supernatant solution was adjusted to 5·2, and the resulting small precipitate was discarded. To the supernatant fluid, acetone was added to 40% concentration, the mixture was allowed to stand for 24 hr, and the resulting precipitate was washed with acetone and ether, and dried as above. The dry

material was suspended in 100-fold its weight of water, the pH of the suspension was adjusted to 5·2, and any insoluble material was discarded. To the supernatant, acetone was added to 40% concentration and the mixture let stand for 24 hr. The resulting precipitate was washed with acetone and ether, and dried as above. The preparation was suspended in 100-fold its weight of water, the pH of the suspension was adjusted to 5·2, and acetone was added to 30% concentration. After allowing the mixture to stand for 2 hr, the resulting precipitate was discarded. Additional acetone was added to the supernatant to a 40% concentration, the mixture was allowed to stand for 4 hr, and the resulting precipitate was washed and dried as above. The light-tan powder thus obtained contained 0·14% copper. It formed a reddish-brown solution in water in contrast to the blue colour of even the very crude preparations of ceruloplasmin (HOLMBERG and LAURELL, 1948).

TABLE 1.—COPPER CONTENT AND YIELD OF SUCCESSIVE COPPER-RICH FRACTIONS OBTAINED IN THE COURSE OF TWO DIFFERENT METHODS FOR THE ISOLATION OF CEREBROCUPREIN I

Method and step in preparation of fraction	Concentration of copper in fraction (%)	Yield per 100 g fresh tissue	
		Solids* (protein) (mg)	Copper (μg)
Method A			
Buffer extract of fresh tissue	0·002	3000†	60
First acetone precipitation	0·007	560†	39
Dialysis, pH 8·5 precipitation	0·034	100	34
Second acetone precipitation	0·061	38	23
Third acetone precipitation	0·094	14	13
Fourth acetone precipitation	0·14	3·6	5·1
Preparative paper electrophoresis	0·24‡; 0·30§	0·9‡	2·1
Method B			
Buffer extract of fresh tissue	0·002	3000†	60
First acetone precipitation	0·010	438†	44
Treatment with $CHCl_3$—CH_3OH, dialysis	0·079	39	31
Second acetone precipitation	0·14	12	17
Preparative paper electrophoresis	0·25*; 0·33‡	1·8‡	5·8

* Based on dry weight except where otherwise indicated.

† Buffer extracts of the fresh tissue contained much dialysable material, some of which was carried down in the first acetone precipitation. Dialysed buffer extracts of the fresh tissue contained about 450 mg of protein per 100 g of fresh tissue.

‡ Based on N 6·25.

§ Based on protein concentration calculated from the area under the sedimentation curve at the time of ultracentrifugation.

Method B: *Treatment with chloroform-methanol at room temperature.* This method was developed following the observation that, when fraction I was treated at room temperature with chloroform-methanol mixture under conditions similar to those used in the preparation of total lipide extracts (FOLCH *et al.*, 1951) from the fresh tissue, its copper remained water-soluble in undialyzable form. The 0·01% copper fraction was suspended in 4-fold its weight of water. To the suspension was added at room temperature 20-fold its volume of 2 : 1 v/v chloroform-methanol mixture. The resulting mixture was stirred for 20 min, and then allowed to stand for 10 min. The insoluble material was collected on a Büchner funnel and washed by suspension in a small volume of chloroform-methanol

followed by filtration through a Büchner funnel. The washing with chloroform-methanol was repeated once, followed by a similar washing with acetone. The total time in contact with organic solvents at room temperature was about 75 min.

The washed material was suspended in 40-fold its weight of water, the suspension stirred for 30 min, then allowed to stand for 10 min, and finally filtered through a Büchner funnel. The clear red filtrate was dialysed against several changes of redistilled water for 90 hr at 4°C. After dialysis, the solution contained about 0·2% solids. To it was added one-tenth its volume of 0·1 M-sodium acetate buffer (pH 5·2) followed by the addition at −10°C., of acetone to 50% concentration. The mixture was allowed to stand for 2 hr, and the resulting precipitate was collected by centrifugation. The wet precipitate was suspended in about 25-fold its volume of water, the suspension was centrifuged, and the small amount of insoluble material discarded. An aliquot of the supernatant solution was dried and found to contain 0·14% copper. This material was green whereas the subfraction with similar copper content prepared by method A appeared tan in the dry state and reddish-brown in aqueous solution. The remainder of the solution was diluted to a concentration of 1% solutes, buffer and acetone were then added to it exactly as described immediately above. This step, however, did not result in significant further increase in copper concentration and therefore has been omitted from Table 1.

Further purification by preparative paper electrophoresis

All analytical paper electrophoresis was carried out by Dr .D. M. SURGENOR, of Harvard Medical School. On paper electrophoresis of the 0·14% copper-containing fraction prepared by method A, two coloured bands, one green and one pink appeared on the unstained strip. The green band migrated toward the anode with a mobility similar to that of a plasma β-globulin. The pink band migrated toward the cathode and was subsequently identified as mainly cytochrome *C* from a study of its oxidized and reduced spectra after elution (carried out by Dr. HELEN HESS, of this laboratory. Densitometer readings after staining for protein indicated that the green band was the main component, and that probably at least three other proteins were present. Copper micro-analysis of similar strips showed the green main component to contain 75% of the total recovered copper, whereas the pink (cytochrome) band contained less than 10.

In view of these results, preparative paper electrophoresis was carried out on 0·14% copper-containing fractions obtained by both methods. The preparations in 10% aqueous solution were placed on strips of Whatman No. 31 paper in amounts not exceeding 50 mg of protein per 2 in. strip width and the electrophoresis run at 200 V for 1-7 hr in the case of the material prepared by method A or for 40 hr in the case of the material prepared by method B. The paper was then cut into bands of 1 to 2 cm length, the first band cut in each case being centred at the zone of maximum green colour (about 2 to 4 cm from the origin) with successive bands then cut in each direction for 10 or 12 cm to include blanks beyond the range of protein migration. Bands of corresponding mobility were pooled in a 15 ml conical tube fitted with a perforated Teflon plug and their protein eluted by centrifugation, followed by washing the papers in each tube three times with 0·1 ml portions of 0·025 M-sodium barbiturate buffer (pH 8·6). The sum total of copper in all the eluted fractions ranged from 70 to 80% of the copper in the material placed on the paper, i.e. there was no evidence that there had been contamination with extraneous copper during electrophoresis. In each run, the material eluted from the pooled bands of maximum green colour accounted for about 50% of the total eluted copper and was hence selected for further investigation. About 10 gm (preparation A) was obtained from the method A 0·14% copper-containing fraction and 12 mg (preparation B) from the method B 0·14% copper-containing fraction.

PROPERTIES

Homogeneity and molecular weight. Ultracentrifugation analyses were carried out by Professor J. L. ONCLEY, of Harvard Medical School. Data were obtained on preparation B at protein concentrations of 1·15, 0·6, and 0·3%. Photographs of the 1·15% run (Fig. 1, curves c and d), indicated that the preparation was about 85% homogeneous. The sedimentation constant was independent of protein concentration within the range studied and had a value of $s_{20,w} = 2{\cdot}9S$, indicating a molecular

weight of the order of 30,000 to 40,000. Preparation A (Fig. 1, curves a and d) at a protein concentration of 0·44% showed the same sedimentation constant and a similar degree of homogeneity. In the case of this preparation, copper analyses after ultracentrifugation established that the main component contained 98% of the recovered copper. Quantitative paper electrophoresis of preparation A (Fig. 2), carried out by Dr. D. M. SURGENOR, also indicated that this preparation was about 85%

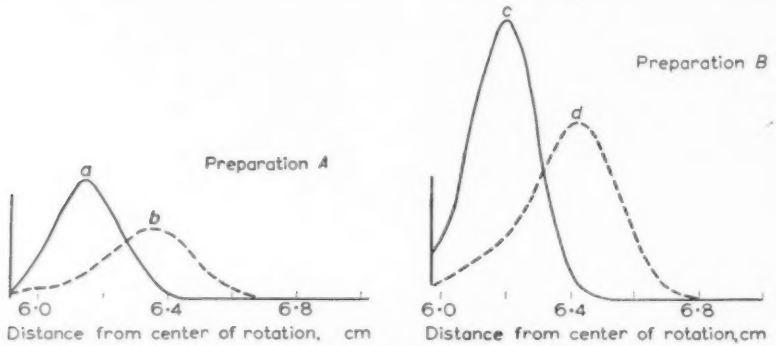


FIG. 1.—Tracings of ultracentrifugation photographs of preparations A and B. Curves a and c after 60 min ultracentrifugation; curves b and d after 120 min ultracentrifugation. Protein concentrations were 0·44% in the case of preparation A and 1·15% in the case of preparation B.

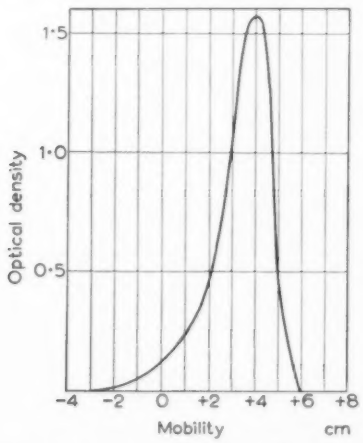


FIG. 2.—Quantitative paper electrophoresis of preparation A. Protein concentration was about 3% and the electrophoresis was run at pH 8·6 in sodium diethylbarbiturate buffer of ionic strength 0·1 on Whatman No. 1 paper at 200 V for 18 hr.

homogeneous and showed it to have an electrophoretic mobility similar to that of a plasma β-globulin.

Copper content. The small amount of purified material available made it difficult to carry out accurate determinations of protein or dry weight from which to compute the precise copper content of cerebrocuprein I. In order to obtain sufficient material in a form suitable for ultracentrifugation analyses, the entire yield of each preparation had been dialysed (against two successive portions of 1000-fold its volume of redistilled water) and copper-free NaCl then added to 0·15 M final concentration.

Aliquots of the resulting solutions were taken for copper and nitrogen analyses, either before or after ultracentrifugation. Protein concentration, and hence copper content of the purified material, was calculated both from the nitrogen values (assuming cerebrocuprein I to be a protein having the usual nitrogen content of 16%) and from the area under the sedimentation curve at the time of ultracentrifugation (assuming a specific refractive index increment of 0·00186 at 577 mμ). Unfortunately, there was not good agreement between the values for protein concentration obtained by these two methods.

Preparation A, before ultracentrifugation, was found to contain 67·0 μg N/μg Cu, a ratio which would correspond to a copper content of 0·24%. After ultracentrifugation, this ratio was 64·5, i.e. it was essentially unchanged, indicating that no contamination with extraneous copper had occurred. However, when protein concentration was estimated from the area under the sedimentation curve, the copper content of this preparation was calculated to be 0·30%.

OL.
1
6/57

Preparation B, after ultracentrifugation, contained 48·7 μg N/μg Cu, a proportion which would correspond to a copper content of 0·33%. However, when an aliquot of this preparation was dialysed (against one portion of 100-fold its volume of redistilled water for 24 hr with occasional shaking), the single dry-weight determination which could be carried out indicated a nitrogen content of 12% and a copper content of 0·25%. Estimation of protein concentration from the area under the sedimentation curve indicated a copper content of 0·30%, a value identical with that obtained by this method of protein estimation in the case of preparation A. If the last value is assumed to be correct and if the less abundant components demonstrated in our preparations by ultracentrifugation are assumed to be copper-free protein material, the copper content of pure cerebrocuprein I would be 0·34%, i.e. 2 atoms of copper per molecule.

The nitrogen content found for preparation B suggested the presence in it of some non-protein material. However, analysis of the parent preparation (the 0·14% copper-containing fraction) showed it to contain less than 1% carbohydrate (as galactose), and presumably lipides (with the possible exception of certain phosphoinositides (LeBaron and Folch, 1957)) would have been removed by the previous treatment of the material with chloroform-methanol. Without further evidence, it is impossible to establish whether cerebrocuprein I does have an unusually low nitrogen content or the observed value is the result of some undetermined source of error.

Absorption spectrum. Both preparations A and B gave pale green aqueous solutions at 0·4 to 1·0% concentration, the former being somewhat yellower, the latter being somewhat bluer in hue. All measurements of absorption spectra on materials prepared by method A were carried out by Dr. Helen Hess. The approximate extinctions ($E^{1\%}_{1cm}$) shown in Fig. 3 are based on the estimates of protein concentration obtained during ultracentrifugation. Comparison of the spectrum of preparation A with that of preparation B shows two noteworthy similarities. First, the spectra of both preparations show a plateau between 600 and 700 mμ although the maximum of this plateau appears to be at about 640 mμ in the case of preparation A and at about 660 mμ in the case of preparation B. Secondly, the spectra of both preparations show less extinction than most proteins in the 270 to 280 mμ range, with absorption at 270 mμ exceeding that at 280 mμ in both cases. The spectrum of preparation A differs strikingly from that of preparation B, however, both in its sharp peak at 405 mμ, and in its failure to show a minimum at about 535 mμ. These

features of the spectrum of preparation A may be attributed to the presence of small amounts of contaminants. Thus, an iron porphyrin-containing protein (such as the cytochrome *C* removed from this preparation by preparative electrophoresis), if present to only about 2% of the total weight of the preparation, would account for the greater absorption at 405 mμ of preparation A as compared to preparation B*. Subtraction of 2% of the extinction ($E^{1\%}_{1cm}$) of cytochrome *C* from the extinction ($E^{1\%}_{1cm}$) of preparation A also gives a difference spectrum showing a minimum in the 530-mμ region. That the absorption peak at 405 mμ is not a true characteristic

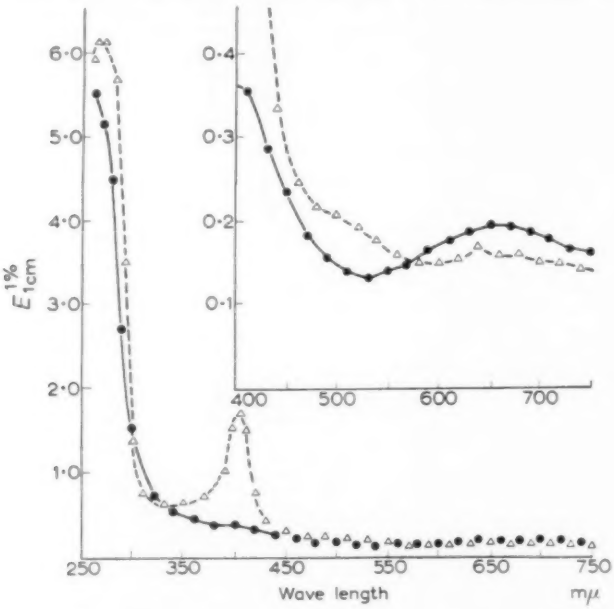


FIG. 3.—Absorption spectra of preparation A (— △ — △ —) and preparation B (— · — · —). Insert shows 400 to 750 mμ range with a 10-fold magnification of the optical density scale. Protein concentrations at the time of measurement were approximately 0·44% in the case of preparation A and 0·60% in the case of preparation B.

of cerebrocuprein I was further shown by comparison of the extinction of preparation A with that of the parent preparation at the 0·06% copper stage; as copper content increased, extinction at 405 mμ was reduced by more than half, whereas extinction at 640 mμ approximately doubled. That the increase in absorption at the latter wavelength was not proportional to the increase in copper content is not surprising in view of the relatively low magnitude of the maximum shown by the purified material together with the finite absorption of other proteins in this region.

Stability of the copper-protein bond. The capacity of the copper in cerebrocuprein I to react directly with sodium diethyldithiocarbamate was tested in the following manner. To 0·1 ml of an approximately 1% solution of preparation B was added 0·2 ml of water, 0·05 ml of 0·1 M-sodium barbiturate buffer (pH 8·6) and 0·05 ml of a 2% solution of sodium diethyldithiocarbamate. The optical density at 445 mμ was measured against a water-barbital-carbamate blank, and the result obtained corrected

* Calculations were based on the values for cytochrome *C* extinction given by PAUL (1951).

VOL 1 1956/

for the already known absorption of the copper protein itself at this wavelength. Under these conditions, all of the copper in cerebrocuprein I appeared to react directly with sodium diethyldithiocarbamate. However, only about 75% of the colour had developed within 15 min, and a stable maximum of colour development was not reached until about 45 min after addition of carbamate. Such a rate of reaction is substantially slower than that exhibited by inorganic copper.

The stability of the copper-protein bond to pH has been investigated both in acetate buffer extracts of the fresh tissue (fraction I) and in the 0·14% copper-containing fraction prepared by method B. Aliquots of the acetate buffer extract corresponding to 18 g of the fresh tissue were each dialysed for 24 hr against 9 volumes of redistilled water and then over two successive 48 hr periods against two successive portions of 9 volumes of copper-free 0·1 M-sodium acetate or citrate-NaOH-HCl buffer, with

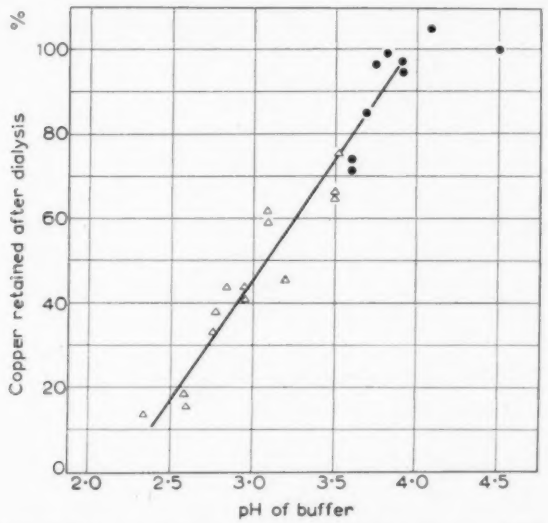


FIG. 4.—Effect of pH on the dialysability of fraction I copper. ●, dialysis against 0·1 M-sodium acetate buffer; △, dialysis against 0·1 M-citrate buffer.

occasional shaking. The contents of the bag were then analysed for copper. The amounts of copper which remained undialysed at different pH's, expressed as percentage of the copper present in the original extract before dialysis, are shown in Fig. 4. It can be seen, first, that fraction I copper remained essentially undialysable at pH's as acid as 3·9. The absence of dialysable copper at pH's 4·5, 4·1, and 3·9 was confirmed by analyses of the three solutions which had been external to each dialysis bag. These external solutions were found to be essentially copper-free. Secondly, Fig. 4 shows the pH of 50% copper loss by dialysis under these conditions to be about 3·1. Over the range between 15% and 85% copper retention, the experimental points are in good agreement with the theoretical hydrogen-ion dissociation curve calculated from the Henderson-Hasselbalch equation for an acidic group with a pK of 3·05. However, since the conditions employed in these experiments would not represent states of reversible equilibrium, it seems probable that the values in Fig. 4 simply represent an effect of pH on the rate at which copper dissociates from the protein.

There was no evidence of selective reduction by citrate of copper-to-protein binding such as has been described for artificial combinations of copper with serum albumin (KLOTZ and FIESS, 1951). The 0·14% copper-containing fraction prepared by method B, lacking only the mild step of preparative paper electrophoresis for final purification, also retained all of its copper on dialysis against acetate buffer at pH 3·9. The essential absence of copper loss on dialysis at this pH was confirmed by analysis of the external solutions, which were found to have a total copper content of less than one-fifth the amount present inside the dialysis bag.

Tests for enzymic activity. Preparations by method A at the second acetone precipitation stage, having a copper content of 0·06%, were tested for enzymic activity by Dr. HELEN HESS. Such preparations, when dissolved in 0·1 M-acetate buffer (pH 6·0) and tested for ability to oxidize *p*-phenylenediamine, showed no evidence of polyphenol oxidase activity. These preparations had some peroxidase activity, but this was not disproportionate to the cytochrome which was later found to be present.

DISCUSSION

The first step in this study was to establish the reproducibility of method A as a procedure for the preparation from brain of a protein material containing 0·14% copper. Additional differential acetone precipitations at a variety of pH's and ionic strengths failed to purify this material further, in particular to free it from the contaminant responsible for its red colour. This apparent impasse was overcome by the use of paper electrophoresis, which made it possible to remove the bulk of the red contaminant, later identified as cytochrome *C*, and to obtain a small amount of a green protein containing 0·24 to 0·30% copper. This copper protein appeared to be about 85% homogeneous, both by ultracentrifugation and by analytical paper electrophoresis, but the study of its properties was severely limited in scope by the small amount of material available. However, enough evidence was gathered to suggest that the copper protein thus obtained was different from any of the previously described copper proteins. At that time, a chance observation showed that the procedure of preparation could be greatly simplified and the yield of copper protein greatly increased by the introduction of treatment with chloroform-methanol, as described in the experimental section under method B. The green protein obtained by this more efficient procedure contained 0·25 to 0·3% copper, was about 85% homogeneous by ultracentrifugation and appeared to have properties identical with those of the previous preparation. Study of this second preparation confirmed that the copper protein isolated from brain did indeed differ from previously described copper proteins, and it is in view of these differences that the name cerebrocuprein I has been coined.

It is possible that the properties of the naturally occurring copper protein have been modified by the rather rigorous procedures employed in its isolation. In this respect, the advantages and disadvantages of the pH 4·5 acetate buffer extraction as an initial step to obtain fraction I brain copper protein(s) from the fresh tissue have been discussed in detail in a previous paper (PORTER and FOLCH, 1957). The observation that the copper in fraction I remained undialysable on acidification down to pH 3·9 seems to indicate that the carrying out of the extraction at pH 4·5 provides a reasonable margin of safety with respect to the effect of pH on the stability of a copper-protein bond. Of course, there remains the possibility that some rearrangement of copper-protein

VOL
1
1956/

linkage may have occurred even though no copper is rendered dialysable. The treatment with chloroform-methanol used in method B is admittedly an extremely drastic procedure to employ in protein isolation. However, the major part of the brain copper in fraction I remained water-soluble and nondialysable after such treatment, and it is clear that treatment with chloroform-methanol is not in itself responsible for the properties of cerebrocuprein I, since a similar sedimentation constant and absorption sprectum were found in material prepared by method A in which the only organic solvent employed was acetone and the temperature of the acetone-water mixtures was maintained at $-10°$. There is also some precedent for such a step in the reports of utilization of procedures of similar character in the isolation of several other copper proteins including ceruloplasmin (HOLMBERG and LAURELL, 1948) which retains enzymic activity following treatment of a similar type. If acceptable on the basis of the above considerations, the treatment with chloroform-methanol had the great advantage of achieving an 8-fold rise in copper concentration with loss of less than one-third of the original copper (Table 1). As a result of this effective step, the yield of the final product obtained by preparative paper electrophoresis in method B was almost 3-fold that obtained in method A.

The amount of cerebrocuprein I isolated by method B accounts for about 10% of the total copper in fraction I, and the amount isolated by method A accounts for only 4%. While these relatively low yields are most likely inherent in the present procedures of isolation, the possibility should be kept in mind that fraction I may contain more that one copper protein. If, however, cerebrocuprein I is the only copper protein present in fraction I, it would account for 40% or more of the total bovine fresh-tissue copper and for over 60% of the total fresh-tissue copper of both human cortex and human white matter (PORTER and FOLCH, 1957).

The comparison of cerebrocuprein I with other copper proteins which have been isolated from mammalian tissues, namely ceruloplasmin (HOLMBERG and LAURELL, 1948) butyryl coenzyme A dehydrogenase (MAHLER, 1954), haemocuprein and hepatocuprein (MANN and KEILIN, 1938), and a purified copper protein from horse liver (MOHAMED and GREENBERG, 1954), reveals both similarities and differences. Thus, in common with most of these other copper proteins, cerebrocuprein I contains 0·3% copper, is bluish-green with an absorption peak between 600 and 700 mμ, and appears to be more resistant than most proteins to the denaturing action of organic solvents. Its molecular weight of 30,000 to 40,000, which corresponds to 2 atoms of copper per molecule, is similar to that of haemocuprein, hepatocuprein, and the copper protein from horse liver, but is only about one-fourth the molecular weight of approximately 150,000 assigned to ceruloplasmin and to butyryl coenzyme A dehydrogenase. It is, of course, possible that cerebrocuprein I as here isolated represents only a fragment of a larger naturally occurring molecule.

On the other hand, cerebrocuprein I appears to differ from previously described mammalian copper proteins both in its absorption spectrum and in the stability of the copper-protein bond to pH and to competing chelating agents. Thus, the absorption spectrum of cerebrocuprein I shows a plateau from 600 to 700 mμ ,with a maximum located at about 660 mμ. Its extinction coefficient, $E^{1\%}_{1cm}$, at this maximum appears to be approximately 0·18. By comparison, ceruloplasmin shows a clear peak at 610 mμ, with an extinction coefficient of 0·68, i.e. more than 3-fold the maximum extinction of cerebrocuprein I in the 600 to 700 mμ range. In this connection, it is of interest

that, when the spectra of artificial combinations of copper with amino acids or proteins (in which copper appears to be bound only to carboxyl groups) are compared with the spectra of similar combinations in which copper is bound to nitrogenous groups, the former show lower extinctions and peaks at longer wavelengths than the latter (KLOTZ, FALLER, and URQUHART, 1950). One may therefore speculate that, if the copper in cerebrocuprein I should prove to be bound to carboxyl groups, this type of linkage might provide an explanation both for its relatively low extinction and for the position of its absorption peak at a relatively long wavelength. In addition, cerebrocuprein I shows relatively little absorption at 280 mμ, a fact which indicates an extremely low tryptophan content. The absorption spectrum gives no evidence of a flavin prosthetic group such as has been described in butyryl coenzyme A dehydrogenase.

The ability of cerebrocuprein I to retain all of its copper on dialysis at a pH as acid as 3·9 is also noteworthy. In contrast, ceruloplasmin (HOLMBERG and LAURELL, 1951) and artificial combinations of copper with serum proteins (GUBLER *et al.*, 1953) both have been reported to lose copper on dialysis at pH 4·5 and a significant portion of the protein-bound copper accumulating in the brains of patients with hepatolenticular degeneration also becomes dialysable at this pH (PORTER and FOLCH, 1957). This failure of cerebrocuprein I to lose copper on prolonged dialysis at a pH as acid as 3·9 suggests that its copper may be bound to a group having a relatively low pK for hydrogen-ion dissociation. However, with the present data, it is not possible to conclude whether the copper loss associated with acidification represents a denaturation of protein, possibly dependent upon a group having a pK in this region, or is a result of the copper having been bound more directly to such a group.

Copper in cerebrocuprein I reacts directly with sodium diethyldithiocarbamate. This reaction is not given by copper in ceruloplasmin or hepatocuprein, the two other mammalian copper proteins in which this point has been studied. Thus, the copper-protein linkage in cerebrocuprein I appears to be less stable to a competing chelating agent but more stable to acidity than is the case in other copper proteins. Its capacity to react directly with sodium diethyldithiocarbamate might raise some question as to whether or not the copper found in this protein from brain represents an artifact of the procedure of isolation. However, that the copper in cerebrocuprein I was originally present in brain tissue was established by a systematic check for contamination at each step in the isolation procedure (as described under Methods). Further evidence that cerebrocuprein I represents a copper-protein present in brain tissue, although possibly isolated in modified form, includes its preparation by two different methods, its characteristic resistance to denaturation by chloroform at an early stage in purification, and the relatively high stability of its copper-protein bond to acidification.

SUMMARY

A protein containing 0·25 to 0·3% copper has been isolated from bovine brain under copper-free conditions. It has been obtained by two different methods as a preparation about 85% homogeneous by ultracentrifugation with a sedimentation constant of 2·9 *S* indicating a molecular weight of approximately 30,000 to 40,000. The copper-containing protein is green with a broad absorption maximum of low extinction at 660 mμ. Its copper reacts directly with sodium diethyldithiocarbamate

VOL
1
1956/

but at a rate slower than that of inorganic copper. The copper in parent fractions remained undialysable at pH's as acid as 3·9.

Acknowledgements—The authors wish to thank Professor J. L. ONCLEY, Dr. D. M. SURGENOR, and Dr. HELEN HESS for helpful discussion as well as for carrying out special procedures as indicated in the text. This investigation was supported by research grants B-156, B-572, and B-921 from the Institute of Neurological Disease and Blindness, of the National Institutes of Health, United States Public Health Service.

REFERENCES

EDEN A. and GREEN H. H. (1941) *Biochem. J.* **34,** 1202.
FOLCH J., ASCOLI I., LEES M., MEATH J. A., and LEBARON F. N. (1951) *J. biol Chem.* **191,** 833.
GUBLER C. J., LAHEY M. E., CARTWRIGHT G. E., and WINTROBE M. M. (1953) *J. clin. Invest.* **32,** 405.
HOLMBERG C. G. and LAURELL C. B. (1948) *Acta Chem. Scand.* **2,** 550.
HOLMBERG C. G. and LAURELL C. B. (1951) *Acta Chem. Scand.* **5,** 476.
KLOTZ I. M., FALLER I. L., and URQUHART J. M. (1950) *J. Phys. Colloid Chem.* **54,** 18.
KLOTZ I. M. and FIESS H. A. (1951) *J. Phys. Colloid Chem.* **55,** 101.
LATNER A. L. (1952) *Biochem. J.* **51,** xii.
LEBARON F. N. and FOLCH J. (1957) *J. Neurochem.* In press.
MAHLER H. R. (1954) *J. biol. Chem.* **206,** 13.
MANN T. and KEILIN D. (1938) *Proc. Roy. Soc.* **B 126,** 303.
MOHAMED M. S. and GREENBERG D. M. (1954) *J. gen. Physiol.* **37,** 433.
PAUL K. G. (1951) in *The Enzymes, Chemistry and Mechanism of Action* (Editors: SUMNER J. B. and HYRBACK K.) Vol. II part I. p. 380. Academic Press, New York.
PETERS J. P. and VAN SLYKE D. D. (1932) *Quantitative Clinical Chemistry Methods* p. 532. Williams and Wilkins, Baltimore.
PORTER H. (1951) *Arch. Biochem. Biophys.* **31,** 262.
PORTER H. and FOLCH J. (1954) *Jed. Proc.* **13,** 275.
PORTER H. and FOLCH J. (1956) *Progress in Neurobiology—I. Neurochemistry* (Editors: KOREY S. R. and NURNBERGER J.) p. 40. Hoeber, New York.
PORTER H. and FOLCH J. (1957) *Arch. Neurol. Psychiat.* In press.

VOL. 1 56/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 272-278. Pergamon Press Ltd., London

THE DISTRIBUTION OF SEROTONIN, 5-HYDROXYTRYPTOPHAN DECARBOXYLASE, AND MONOAMINE OXIDASE IN BRAIN*

DONALD F. BOGDANSKI, HERBERT WEISSBACH, AND SIDNEY UDENFRIEND
Laboratories of Chemical Pharmacology and Clinical Biochemistry, National Heart Institute, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland

(*Received* 28 *December* 1956)

THE presence of serotonin in brain was first demonstrated by bioassay techniques (TWAROG and PAGE, 1953; AMIN, CRAWFORD, and GADDUM, 1954). Its presence in the central nervous system was subsequently corroborated by spectrophotofluorometric procedures in conjunction with counter-current distribution (BOGDANSKI, PLETSCHER, BRODIE, and UDENFRIEND, 1956). Enzymes involved in the formation and destruction of serotonin are widely distributed in organ tissues and have also been found in brain (CLARK, WEISSBACH, and UDENFRIEND, 1954; SJOERDSMA, SMITH, STEVENSON, and UDENFRIEND, 1955). Evidence is now accumulating that serotonin plays some important role in brain function (GADDUM, 1953; WOOLLEY and SHAW, 1954; BRODIE, PLETSCHER, and SHORE, 1955), although the exact nature of its central role remains to be determined. The present studies on the distribution of serotonin, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in various parts of the brain, and the findings concerning their intracellular localization, were designed to provide additional information which may help in determining the function of this recently discovered compound.

METHODS

Serotonin assay. The methods for assaying brain serotonin have been previously reported by BOGDANSKI, PLETSCHER, BRODIE, and UDENFRIEND, (1956). Brains from dogs and cats were used in the present investigations. Animals were lightly anesthetized with sodium pentobarbital and then killed by the intravenous injection of air, though in some cases the animals were sacrificed without previous anesthetization. The brains were removed as rapidly as possible, dissected at 4°C, and the various parts were weighed and homogenized in 0·1 N-HCl. The concentration of tissue in the homogenate was usually 333 mg/ml. More dilute homogenates were prepared where the amount of tissue was small. In all cases spectrophotofluorometric purity was assured by determining the peaks of activation and fluorescence, which for serotonin in 3 N-HCl are 295 mμ and 550 mμ respectively. To assay serotonin in some parts of the brain, such as the septal areas, it was necesssary to combine material from more than one animal.

Monoamine oxidase determination. Monoamine oxidase determinations were carried out by measuring serotonin disappearance as previously reported by SJOERDSMA, SMITH, STEVENSON, and UDENFRIEND (1955). Inhibition by iproniazid, an inhibitor of monoamine oxidase in homogenates, was used as a criterion of enzyme specificity. Usually, 1 g of tissue was homogenized with 2 ml of distilled water. When tissue samples were less than 0·5 g, 1-2 ml of water was used so that there would be a sufficient volume of homogenate. Homogenate concentrations, therefore, varied from 333 mg/ml to approximately 150 mg/ml. To 1 ml of homogenate in a beaker were added 1 ml of

* Presented in part before the Society for Pharmacology and Experimental Therapeutics, Iowa City, Iowa, September 1955.

VOL
1
1956/

serotonin solution (4 μmol/ml), 0·3 ml phosphate buffer (pH 7·0), and 0·7 ml of water. The beakers were then incubated in a metabolic shaker at 37°C for 15 and 30 min. Samples containing inhibitor (iproniazid phosphate 10^{-3} M) were pre-incubated for 15 min before the addition of substrate. Experimental and control samples were incubated without substrate for a similar period of time. The reaction rate was found to be essentially linear up to 30 min. The complete inhibition of serotonin disappearance by iproniazid*, was evidence of the specificity of the assay for monoamine oxidase. Results are reported as micrograms of serotonin destroyed per gram of tissue per hour.

5-Hydroxytryptophan (5HTP) decarboxylase determination. The method of assay of 5HTP decarboxylase was previously described by CLARK, WEISSBACH, and UDENFRIEND (1954). A sample of homogenate 1 ml, prepared as for monoamine oxidase studies, was incubated at 37°C in a metabolic shaker with 0·3 ml of 1 M-PO_4 buffer (pH 8·1), 1 μmol of 5HTP, *p*-tolyl choline ether in a concentration of 10^{-2} M, and 0·1 ml octyl alcohol. Pre-incubation with monoamine oxidase inhibitors was carried out for 15 min before addition of substrate. Control experiments included incubation with serotonin to determine the efficiency of monoamine oxidase inhibition. Combination of these two inhibitors prevented any detectable loss of serotonin during the time of incubation, so that serotonin formation could be used as a measure of 5HTP decarboxylase activity. The reactions were stopped by transferring a 1 ml aliquot of incubation mixture to a 60 ml glass-stoppered bottle and extracting according to the procedure for serotonin assay.

The quantity of serotonin produced was found to be proportional to the amount of enzyme used. With rabbit and dog brain the rate of reaction was essentially independent of 5HTP concentration up to 4 μM. This is in contrast to the marked substrate inhibition reported by GADDUM and GIARMAN (1956). Time curves indicated that the rate of formation of serotonin by rabbit brain decarboxylase is linear up to 60 min. Incubation with substrate was carried out for 30 min. Results are reported as micrograms of serotonin formed per gram of tissue per hour.

Fractionation of brain and gastric mucosa. Rabbit brain and gastric mucosa† were fractionated according to the method of HOGEBOOM and SCHNEIDER (1950), after homogenizing in 0·88 M-sucrose. The low-speed sediment (nuclei and cell debris) and the high-speed sediment ("microsomes") were found to contain little mitochondrial contamination, as shown by succinic dehydrogenase assay. To prevent serotonin destruction during tissue preparation the animals were given (150 mg/kg) iproniazid 60 min before they were sacrificed. Aliquots (1 mg) of the homogenate fractions were taken for enzyme studies and the remainder was used for serotonin analyses.

RESULTS

Distribution in brain. The results of the distribution studies on dogs are summarized in Table 1, which gives the quantities of serotonin, 5HTP decarboxylase, and monoamine oxidase in various parts of the brain.

The serotonin concentration and 5HTP decarboxylase activity were found to vary markedly from one part of the brain to another, and, except in the pyriform cortex and amygdala, it appears that they parallel each other. Caudate nucleus was also found to contain decarboxylase activity disproportionate to its serotonin content. Serotonin concentrations in the fornix, optic tracts, lateral geniculate bodies, medial geniculate bodies, and white matter taken from the corpus callosum and internal capsule were so low that clear-cut fluorescence peaks were not observed and readings above the blank were small. It is of interest that 5HTP decarboxylase activity was also extremely low in these areas.

On the other hand, monoamine oxidase activity, with the exception of the hypothalamus and white matter, was found to be relatively evenly distributed. A comparison of Tables 1 and 2 reveals that the distributions of serotonin in cat and dog brains are essentially similar, in so far as has been determined.

* Inhibition was also achieved with other known inhibitors of monoamine oxidase, octyl alcohol, *p*-tolyl choline ether.

† The mucosa was unavoidably contaminated with smooth muscle.

TABLE 1.—THE DISTRIBUTION OF SEROTONIN, 5HTP DECARBOXYLASE, AND MONOAMINE OXIDASE IN DOG BRAIN

Tissue*	Number of animals	Serotonin content (μg/g)	Number of animals	5HTP decarboxylase activity (μg/5HT formed per g/hr)	Number of animals	Monoamine oxidase activity (μg/5HT destroyed per g/hr)
Amygdala	6	2·1 ± 0·4	4	18 ± 2	4	968 ± 23
Hypothalamus	7	1·7 ± 0·3	5	117 ± 25	6	1624 ± 510
Septal region	4	1·5 ± 0·4	4	109 ± 24	4	1212 ± 58
Midbrain	3	1·0 ± 0,2	3	98 ± 7	3	842 ± 122
Pyriform cortex	4	0·94 ± 0·08	4	16 ± 1	4	926 ± 183
Caudate nucleus	9	0·72 ± 0·2	6	306 ± 37	4	935 ± 200
Medulla	3	0·62 ± 0·2	3	32 ± 3	3	1117 ± 230
Thalamus	6	0·57 ± 0·07	3	38 ± 7	3	940 ± 164
Hippocampus	3	0·64 ± 0·04	4	16 ± 3	4	1176 ± 38
Pons	3	0·38 ± 0·1	2	28 ± 7	3	936 ± 110
Olfactory bulb	3	0·35 ± 0·04	1	5	1	573
Cortical grey matter[1]	3	0·27 ± 0·04	4	7 ± 2	4	819 ± 300
Cortex[2]	2	0·17 ± 0·08	—	—	—	—
Cerebellum	3	<0·09	3	<9	3	930 ± 87
Corpus callosum + internal capsule[3]	4	—	4	4 ± 1	4	466 ± 120
Lateral geniculate[3]	4	—	4	8 ± 3	4	844 ± 95
Medial geniculate[3]	4	—	4	7 ± 1	4	—
Fornix[3]	4	—	4	9 ± 2	4	707 ± 158
Optic tract[3]	4	—	4	7 ± 3	4	701 ± 95

* Where the quantities of tissue were small, tissues from two animals were combined.
[1] Grey matter taken at random from the neocortex.
[2] A piece of tissue containing both grey and white matter.
[3] Even after combining tissues from two animals, no 550 mμ fluorescence peaks could be detected. Values are given ± the standard error of the mean.

Intracellular localization of serotonin, 5HTP decarboxylase, and monoamine oxidase. Separation of the various cellular components is difficult to accomplish owing to the large amount of lipid and cytoplasmic components found in nerve cells. Furthermore, the amounts of serotonin found in the central nervous system are small. For these reasons the fractionation studies reported here were carried out with gastric mucosa, a tissue rich in serotonin. However, the results of preliminary experiments with brain were found to be essentially in agreement with those obtained with gastric mucosa. The distribution of serotonin in the cellular fractions of gastric mucosa are shown in Table 3. Essentially all the serotonin originally in the tissue was accounted for after fractionation. The results indicate that some serotonin is associated with each particulate fraction and that it does not appear to be specifically localized in any one fraction. The serotonin that is associated with the various particles is apparently not there merely as an artifact of homogenization. This was

VOL 1 1956/

TABLE 2.—THE DISTRIBUTION OF SEROTONIN IN CAT BRAIN

Tissue	Serotonin (μg/g*)
Hypothalamus	2·0 ± 0·3
Midbrain	1·7 ± 0·5
Caudate nucleus	1·6 ± 0·1
Medulla	1·2 ± 0·5
Thalamus	1·1 ± 0·3
Pons	0·70 ± 0·3
Grey matter (cortex)	0·68 ± 0·1
Cerebellum	0·27 ± 0·5
Cortex†	0·24
White matter (cortex)	<0·13

* Each value represents the average of three separate determinations presented with the standard error of the mean.
† A piece of tissue containing both grey and white matter (one sample).

TABLE 3.—INTRACELLULAR DISTRIBUTION OF SEROTONIN IN RABBIT GASTRIC MUCOSA

Fraction	Serotonin per cent of total
Low-speed sediment (nuclei + cell debris)	28
Medium-speed sediment (mitochondria)	29
High-speed sediment (microsomes)	26
Soluble fraction	19

shown by an experiment on brain in which additional serotonin was added to the sucrose before homogenization when all the added serotonin was found in the high-speed supernatant.

As in other tissues (CLARK, WEISSBACH, and UDENFRIEND, 1954), the 5HTP decarboxylase of brain was found to be present almost entirely in the soluble fraction. This is in agreement with the finding of GIARMAN (1956). In preliminary studies with monoamine oxidase almost all of the activity was found associated with cell particles.

Serotonin in human brain. Preliminary studies have shown that serotonin is also present in human brain, where it was found in larger amounts in the hypothalamus and thalamus than in the cortex. The value obtained for hypothalamus was about 0·5 μg/g. However, since tissues were obtained several hours *post mortem*, enzymic destruction had no doubt taken place.

DISCUSSION

Values for brain serotonin presented here are higher than those reported by other investigators (TWAROG and PAGE, 1953; AMIN, CRAWFORD, and GADDUM, 1954). Since the specificity of the spectrophotofluorometric and bioassay procedures has been demonstrated, the relative efficiency of the extraction procedures may

account for the observed differences. With the butanol extraction procedure, 90–100% of added serotonin can be accounted for in brain homogenates. Using acetone extraction (20 volumes), TWAROG and PAGE (1953) were able to achieve only 60% recovery from brain. AMIN, CRAWFORD, and GADDUM (1954) used extraction with 20 volumes of acetone, but did not report recovery data on brain samples. CORREALE (1956) has also reported poor recoveries of serotonin from brain using extraction with 20 volumes of acetone. In our laboratory such acetone extraction procedures have been found to yield low and variable recoveries.

The present findings also differ from those of GADDUM and GIARMAN (1956) in that much more 5HTP decarboxylase activity was observed in brain tissues. However, the present data are based on initial maximal rates of reaction. During the interval taken (30 min), the time curve of serotonin production by brain was shown to be essentially linear. Substrate inhibition, reported by GADDUM and GIARMAN (1956), was not encountered in these studies.

The ubiquitous distribution of monoamine oxidase may signify that, in addition to a role at specific sites, it may also be involved in protecting the brain from serotonin and other free amines. It is important to consider the possibility of there being more than one monoamine oxidase, one of which may be more specific for serotonin. This would be comparable to the relatively specific and nonspecific enzymes involved in acetylcholine metabolism.

It is evident that serotonin and 5HTP decarboxylase are present to the greatest extent in the brain stem and in certain subcortical areas of the cerebral hemispheres. In most parts, serotonin concentration and decarboxylase activity show a close correlation. Differences in their relative proportions are found in only a few areas (amygdala and caudate nucleus), suggesting that the rates of turnover of serotonin are not uniform throughout the brain.

Serotonin in the telencephalon appears to be distributed in grey masses within the primitive rhinencephalon. The olfactory bulbs, representing primary olfactory pathways, contain small quantities of serotonin and little decarboxylase. Intermediate amounts of serotonin are found in the neostriatum, which also shows the highest decarboxylase activity found so far. The fact that this structure also has very high acetylcholine synthesizing activity (FELDBERG and VOGT, 1948) probably has functional significance.

The distributions of serotonin and 5HTP decarboxylase follow masses of grey matter along the polysynaptic descending pathways described by GLOOR (1955a,b) which originate in the amygdala and ultimately converge on the hypothalamus through several pathways involving the pyriform cortex, hippocampus, and the septal nuclei. The amygdala are considered to be relay stations between the cortex and hypothalamus. This pattern of distribution of serotonin also corresponds to the rhinencephalic systems 2 and 3 of PRIBRAM and KRUGER (1954), which have functions other than olfactory discrimination. Stimulation and ablation studies involving the rhinencephalon have indicated its importance in modulating autonomic and somatic activity during behavioural reactions, in conditioned reflex responses, and in pathology (for references consult HESS, 1954; KAADA, 1951; KAADA, ANDERSON, and JANSEN, 1954; PRIBRAM and KRUGER, 1954; GLOOR, 1955a,b). Many of the effects described, including mydriasis, salivation, hypertension, tachycardia, mastication, and licking movements, catatonia, fear in dogs and sham rage in cats, have

VOL 1 1956/

been produced when brain serotonin levels were increased through the administration of 5HTP (BOGDANSKI, WEISSBACH, and UDENFRIEND, 1956, 1957). These effects were found to resemble those obtained with lysergic acid diethylamide, and it is of further interest that the actions of the tranquillizers, reserpine, and chlorpromazine have been reported to involve these same areas of the brain (KILLAM, 1956).

The points summarized below are consistent with the thesis that serotonin is important central agent, and, in addition, suggest the nature of its role: (1) Both serotonin and the enzyme involved in its synthesis are normally localized in areas of the brain which are involved in functions modulating autonomic and somatic activities of emotional expression, (2) serotonin through administered 5HTP influences these same functions, (3) tranquillizing drugs also act on these same areas, and (4) the central effects of serotonin seen after administration of 5HTP resemble those of the psychotomimetic agent lysergic acid diethylamide.

VOL. 1 56/57

SUMMARY AND CONCLUSIONS

1. Serotonin, 5HTP decarboxylase, and monoamine oxidase have been determined in various parts of dog and cat brain.

2. Serotonin was found in highest concentrations in brain stem structures, rhinencephalic structures, and in the neostriatum.

3. The distribution of 5HTP decarboxylase paralleled that of serotonin except for the pyriform cortex and amygdala. The caudate nucleus was found to contain the highest decarboxylase activity.

4. Monoamine oxidase was found to be relatively evenly distributed throughout the brain, being somewhat higher in the hypothalamus and lower in white matter.

5. While much of the serotonin in brain is associated with particulate fractions, it is not localized in any one fraction. Brain 5HTP decarboxylase is a soluble enzyme; monoamine oxidase activity is associated with particulate fractions.

6. The implications of the distribution of serotonin to its possible central functions is discussed.

REFERENCES

AMIN A. H., CRAWFORD T. B. B., and GADDUM J. H. J. (1954) *J. Physiol.* **126,** 596.

BOGDANSKI D. F., PLETSCHER A., BRODIE B. B., and UDENFRIEND S. (1956) *J. Pharm. exp. Therap.* **117,** 82.

BOGDANSKI D. F., WEISSBACH H., and UDENFRIEND S. (1956) *Fed. Proc.* **15,** 402.

BOGDANSKI D. F., WEISSBACH H., and UDENFRIEND S. *J. Pharm. exp. Therap.* (manuscript in preparation).

BRODIE B. B., PLETSCHER A., and SHORE P. A. (1955) *Science* **122,** 968.

CLARK C. T., WEISSBACH H., and UDENFRIEND S. (1954) *J. biol. Chem.* **210,** 139.

CORREALE P. (1956) *J. Neurochem.* **1,** 22.

FELDBERG W. and VOGT M. (1948) *J. Physiol.* **107,** 372.

GADDUM J. H. J. (1953) *Ciba Foundation Symposium, London.*

GADDUM J. H. J. and GIARMAN N. (1956) *Brit. J. Pharm. and Chemotherap.* **11,** 88.

GIARMAN N. (1956) *Fed. Proc.* **15,** 428.

GLOOR P. (1955a) *EEG. and Clin. Neurophysiol.* **7,** 223.

GLOOR P. (1955b) *EEG. and Clin. Neurophysiol.* **7,** 243.

HESS W. (1954) *Diencephalon: Autonomic and Extrapyramidal Functions* Grune and Stratton, New York.

HOGEBOOM G. H. and SCHNEIDER W. C. (1950) *J. biol. Chem.* **183,** 123.

KAADA B. R. (1951) *Acta Physiol. Scand.* **24,** supp. 83.
KAADA B. R., ANDERSON P., and JANSEN J. (1954) *Neurology* **4,** 48.
KILLAM E. *Ann. N.Y. Acad. Sci.* In press.
PRIBRSM K. H. and KRUGER C. (1954) *Ann. N.Y. Acad. Sci.* **58,** 109.
SJOERDAMA A., SMITH T. E., STEVENSON T. D., and UDENFRIEND S. (1955) *Proc. Soc. exp. Biol. Med. N.Y.* **89,** 36.
TWAROG B. M. and PAGE I. H. (1953) *Amer. J. Physiol.* **175,** 157.
WOOLLEY D. W. and SHAW E. (1954) *Proc. Nat. Acad. Sci., U.S.* **40,** 228.

VOL.
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 279–288. Pergamon Press Ltd., London

BIOCHEMICAL AND PHYSIOLOGICAL DIFFERENTIATION DURING MORPHOGENESIS—XXI

EFFECT OF HYPOTHYROIDISM AND HORMONE THERAPY ON ENZYME ACTIVITIES OF THE DEVELOPING CEREBRAL CORTEX OF THE RAT*

MAX HAMBURGH† and LOUIS B. FLEXNER

Department of Anatomy, Albert Einstein College of Medicine, New York
and Department of Anatomy and Institute of Neurological Sciences
School of Medicine, University of Pennsylvania, Philadelphia

VOL. 1 56/57

(*Received* 5 *December* 1956)

THE studies reported here were undertaken with the following aims: (1) to follow the change in activity of several enzymes in the frontal cerebral cortex of the rat during its early postnatal life; (2) to determine the effect of hypothyroidism on the developmental pattern of these enzymes; and (3) to test the reversibility of enzyme changes observed in the cretinous animal by instituting hormone-replacement therapy at various periods after thyroidectomy.

These three aims have a considerable background. Studies on the developing frontal cerebral cortex of the foetal guinea pig have demonstrated that there is a well-defined period between the forty-first and forty-fifth days of gestation, during which the activities of several enzymes begin sharply to increase to the adult level (FLEXNER and FLEXNER, 1948; FLEXNER, BELKNAP, and FLEXNER, 1953). We have been interested in extending these observations to the cerebral cortex of the rat in order to test the degree to which this pattern of enzyme change accompanies development in another species. Observations on rats made cretinous at or shortly after birth have shown that behaviour is abnormal (EAYRS, 1953; EAYRS and LISHMAN, 1955), myelination is retarded (BARRNETT, 1943), perikarya of nerve cells are smaller and more closely packed (EAYRS and TAYLOR, 1951), and, apparently, the dendritic fields, number of axons (EAYRS, 1955; EAYRS and HORN, 1955), and capillary bed (EAYRS, 1954) are reduced. We are not aware, however, of any studies on the enzyme activities of the nervous system of the immature cretinous animal, and consequently information on the integrity of systems concerned with metabolism and function is lacking. Cretinous rats treated with thyroxin as late as the twenty-fourth day *post partum* have been shown to recover a normal number of fibres as visualized with silver impregnation (HORN, 1955) and to improve markedly in their behaviour (EAYRS, 1953). The effect of hormone therapy on enzyme activity provides a further test of the reversibility of the changes which accompany cretinism in the rat.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service.

† Post-doctoral Fellow, National Foundation for Infantile Paralysis, 1953–55.

It is to be hoped that an analysis of the factors which govern recovery of the central nervous system of the immature cretinous rat will contribute to an understanding of the problem in the cretinous human infant. In the cretinous human infant the picture has been much confused by the difficulty of assessing the many variables (the level of maternal thyroid function during pregnancy, the rate of passage of thyroid hormone across the placenta, the time of starting treatment and its adequacy, the mental potential of infant) which may determine the mental response of the infant to therapy. In those infants who fail to respond it is believed by some that irreparable damage occurs to the brain at a critical period in development because of lack of hormone and before hormone therapy is instituted; by others that a congenital deficiency of the brain is present, associated with but not caused by hypothyroidism.

The enzymes chosen for study in this investigation were the glycolytic enzyme, aldolase, the aerobic enzymes, succinic dehydrogenase, and cytochrome oxidase, and finally, cholinesterase. In the normal animal it has been found that the first three of these begin rapidly to increase to the adult level of activity at about the tenth day *post partum*. Thyroidectomy shortly after birth decreases the activity of succinic dehydrogenase and cholinesterase, but is without effect in the others. Succinic dehydrogenase activity in thyroidectomized animals is maintained at normal levels if hormone therapy is started at the tenth day, but fails to recover under the conditions of our experiments if therapy is delayed until the fifteenth or twentieth day.

VOL
1
1956/

MATERIALS AND METHODS

Rats of the Wistar strain were used for the two sets of experiments reported here. The first set was designed to follow during early postnatal development the normal patterns of enzyme activity and their response to hypothyroidism. Litters were reduced to six to diminish competition for the mother's milk. Three littermates were kept as controls; three were thyroidectomized by a single subcutaneous injection of 200 μc of carrier-free I^{131} (GOLDBERG and CHAIKOFF, 1949). Frontal cerebral cortex was taken for enzyme assays at 5-day intervals from 1 to 30 days. Animals were sacrificed by decapitation. Frontal cerebral cortex was removed from the underlying white matter by scissors and homogenized in microhomogenizers. Fresh homogenates were used for enzyme assays, except for cytochrome oxidase determinations, where brains were frozen on dry ice and kept in the deep freeze for 2 months prior to homogenization. In the second set of experiments, Wistar rats from a different supplier were used. Each female was permitted to raise eight littermates. Two littermates served as controls; the remainder were thyroidectomized with 200 μc of I^{131} on the day of birth. After various periods without replacement therapy, half of the surviving thyroidectomized animals were given daily a subcutaneous injection of L-thyroxin sodium or triiodothyronine for 15 consecutive days or longer. Treatment was started in one group on the tenth day of age; in the second on the fifteenth day, and in the third on the twentieth day. Each injection contained 2 μg of L-thyroxin sodium or triiodothyronine. Animals in Group I were sacrificed at 25 days of age; in group II at 30 days of age, except two animals which were sacrificed respectively at 65 and 75 days of age after 50–60 days of treatment; and in group III at 35 days of age.

The activity of succinic dehydrogenase was determined on homogenized tissue by the method of QUASTEL and WHEATLEY (1938) as slightly modified by FLEXNER and FLEXNER (1946). In this method an excess of ferricyanide serves as final electron-acceptor from succinate and the reaction rate is limited by the activity of the dehydrogenase. The method has been validated against that in which an excess of cytochrome *c* is added to make the dehydrogenase the limiting factor (FLEXNER and FLEXNER, 1946). Cytochrome oxidase was determined by COOPERSTEIN and LAZAROW'S (1951) procedure in which rate of oxidation of reduced cytochrome *c* is followed spectrophotometrically after addition of homogenized tissue. Cholinesterase was measured by AUGUSTINSSON'S (1948) manometric method, based on CO_2 production from a bicarbonate buffer; acetylcholine was used as substrate. The assay of aldolase depended upon the methods of DOUNCE and BEYER (1948), SIBLEY and LEHNINGER (1949). and LOWRY, ROBERTS, WU, HIXON, and CRAWFORD (1943), as previously described (FLEXNER,

GREENBLATT, COOPERMAN, and FLEXNER, 1956). The reaction mixture consisted of the magnesium salt of fructose-1,6-diphosphate, hydrazine sulphate, and homogenized tissue. After running the reaction for 15 min, as previously described, triosephosphate was converted to acetaldehyde with hot concentrated sulphuric acid and the acetaldehyde determined by colour formation with *p*-hydroxydiphenyl.

RESULTS

1. *Completeness of thyroidectomy and general response to replacement therapy.* Histological examination of the anterior neck region in selected instances confirmed the observations of GOLDBERG and CHAIKOFF (1949) that total thyroid destruction can be achieved within a week by 200 μc of I^{131}; indeed, in the single specimen studied 4 days after injection of I^{131} the parenchyma of the gland was destroyed. By the fifteenth day the thyroidectomized animal could be clearly recognized by the changes in appearance and behaviour so frequently described as characteristic of the cretin (EAYRS, 1953).

As shown in Table 1, there was considerable mortality among thyroidectomized rats with and without replacement therapy. This is in contrast to the much more favourable experience of GOLDBERG and CHAIKOFF (1949), who segregated control and thyroidectomized animals. In those animals which received treatment beginning at the tenth day, weight-gain was greater than in the untreated littermates but somewhat below the controls; in those animals which received treatment beginning at 15 or 20 days, weight-response was less consistent.

TABLE 1.—VIABILITY OF CONTROL, OF THYROIDECTOMIZED AND OF THYROID-DEFICIENT RATS RECEIVING REPLACEMENT THERAPY AT DIFFERENT AGES.

	Number of animals at beginning of replacement therapy			Survivors at termination of therapy as per cent original number of animals		
Group	*C*	*T*	*TR*	*C*	*T*	*TR*
I	20	18	19	90	44	63
II	18	17	18	89	41	55
III	19	21	17	94	19	23

C = Controls; *T* = Thyroidectomized at birth; *TR* = Thyroidectomized animals with replacement therapy. Group I, replacement therapy started at 10 days of age and continued for 15 days. Group II, replacement therapy started at 15 days of age, continued for 15 days in six animals, and in two animals for 50 and 60 days respectively. Group III, replacement therapy started at 20 days of age and continued for 15 days.

2. *Normal pattern of enzyme change during postnatal development.* As shown in Fig. 1, the activity of succinic dehydrogenase is at a relatively low level until the tenth day, when it begins rapidly to increase to a level characteristic of more mature animals. Much the same pattern is followed by cytochrome oxidase (Fig. 2), the tenth day again marking the time when enzyme activity begins sharply to increase. The activity of

aldolase (Fig. 3) follows this pattern less clearly, since there is a substantial rate of increase in activity from birth to the tenth day. The rate of change from the tenth to the fifteenth days, however, is approximately five times the maximum rate observed before the tenth day. Cholinesterase activity (Fig. 4) clearly differs in its characteristics. Here the most rapid increase in activity occurs before the tenth day.

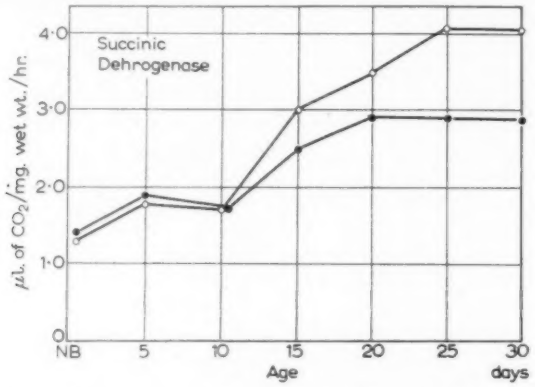


FIG. 1.—Succinic dehydrogenase activity of fresh homogenates of frontal cerebral cortex of normal and thyroidectomized animals.
N.B. = newborn. Each point represents the mean value of several determinations obtained from different litters. (See Table 2.)
○ = control; ● = thyroidectomized

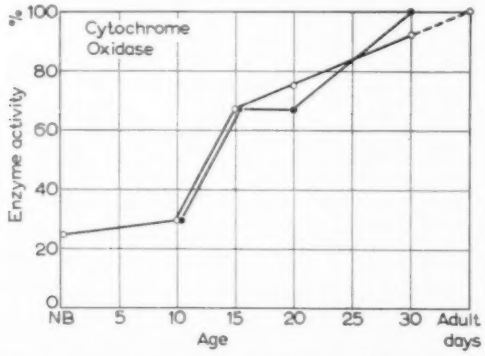


FIG. 2.—Cytochrome oxidase activity of homogenates of frontal cerebral cortex of normal and thyroidectomized animals. Activity is expressed as per cent adult value. All samples of cortex were kept frozen for 2 months before use. Each point represents the mean value of several determinations. (See Table 2.)
○ = control; ● = thyroidectomized

3. *Pattern of enzyme change in thyroidectomized animals.* Thyroidectomy at birth leads to a statistically significant depression of succinic dehydrogenase activity (Fig. 1 and Table 2) first evident in our series at 15 days and sustained for the duration of the observations to 30 days. The mean percentile depression in the thyroidectomized animals varied from 17% at 15 days to 30% at 30 days.

Cholinesterase activity is also depressed by thyroidectomy at birth (Fig. 4 and Table 2). In our series this depression is first evident at the tenth day and is sustained for the duration of the observations. The depression is less marked than with succinic

OL.
1
6/57

TABLE 2.—ENZYME ACTIVITIES OF FRONTAL CEREBRAL CORTEX OF NORMAL AND THYROIDECTOMIZED RATS AS A FUNCTION OF AGE

N = normal controls. T = thyroidectomized animals. The mean and its standard error are given; in parentheses is the number of animals in each group. P = probability of obtaining by random sampling the differences in means of control and thyroidectomized animals (t-test). The unit for succinic dehydrogenase (s.dehase) activity and cholinesterase (ch.e.) is μl CO_2 per mg wet weight per hour; for aldolase and cytochrome oxidase (cyt. oxid.), optical density.

Enzyme	Birth to 1 day		5 days		10 days			15 days		
	N	*T*	*N*	*T*	*N*	*T*	*P*	*N*	*T*	*P*
s. dehase	1·27 ± 0·06(10)	1·40 ± 0·06(6)	1·78 ± 0·14(7)	1·93 ± 0·17(6)	1·73 ± 0·07(5)	1·71 ± 0·13(4)		3·07 ± 0·07(5)	2·51 ± 0·15(5)	<0·05
aldolase	0·16 ± 0·02(6)		0·26 ± 0·03(6)		0·35 ± 0·04(7)	0·37 ± 0·02(2)		0·90 ± 0·06(9)	0·91 ± 0·10(5)	
cyt. oxid	0·06 ± 0·00(2)				0·07 ± 0·01(2)	0·07 ± 0·00(2)		0·16 ± 0·00(4)	0·16 ± 0·01(5)	
ch. e.	0·95 ± 0·04(5)		1·88 ± 0·16(7)	1·91 ± 0·15(6)	3·39 ± 0·10(5)	2·93 ± 0·06(5)	<0·01	3·57 ± 0·06(3)	3·41 ± 0·10(3)	>0·1

Enzyme	20 days			25 days		30 days			35–40 days		
	N	*T*	*P*	*N*	*T*	*N*	*T*	*P*	*N*	*T*	*P*
s. dehase	3·48 ± 0·10(9)	2·95 ± 0·16(7)	<0·01	4·11(1)	2·90(1)	4·08 ± 0·05(3)	2·90 ± 0·10(4)	<0·01			
aldolase	1·04 ± 0·04(4)	1·00 ± 0·08(3)		1·15 ± 0·03(4)	1·08 ± 0·10(2)	1·11 ± 0·04(4)			1.34 ± 0·04(6)	1·24 ± 0·02(4)	>0.1
cyt. oxid	0·18 ± 0·02(3)	0·16 ± 0·02(3)				0·22 ± 0·00(2)	0·25(1)				
ch. e.	3·78 ± 0·07(3)	3·24 ± 0·20(3)	0·05	4·40 ± 0·30(2)	3·90 ± 0·30(2)	4·49 ± 0·40(2)	3.99 ± 0·20(2)		4·96(1)	3·64(1)	

dehydrogenase, averaging about 15%. Most of the groups are too small to demonstrate a statistically significant difference between control and hypothyroid animals. It will be noted, however, that the thyroidectomized animals on the tenth day and thereafter invariably had a lower mean level of activity than the controls. Consistent with this, the mean of the control subgroups at and following the tenth day (sixteen animals) is significantly different at the 1% level from the mean of the corresponding thyroidectomized animals.

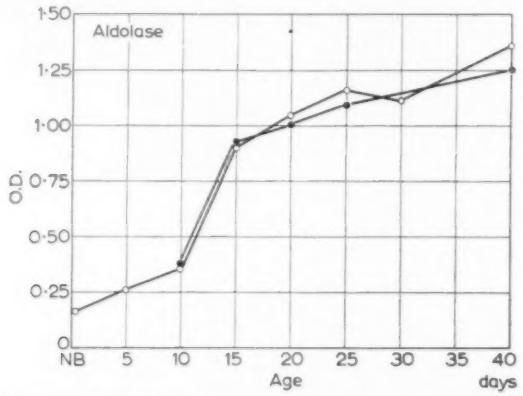


FIG. 3.—Aldolase activity of fresh homogenates of frontal cerebral cortex of normal and thyroidectomized animals. Activity is expressed in terms of optical density (O.D.) per mg wet weight of tissue. Each point represents the mean value of several determinations obtained from different litters. (See Table 2.)

○ = control; ● = thyroidectomized

FIG. 4.—Cholinesterase activity of fresh homogenates of frontal cerebral cortex of normal and thyroidectomized animals. Each point represents the mean value of several determinations obtained from different litters. (See Table 2.)

○ = control; ● = thyroidectomized

Hypothyroidism was without significant effect on the activities of both cytochrome oxidase (Fig. 2 and Table 2) and aldolase (Fig. 3 and Table 2).

4. *Effect of replacement therapy on activity of succinic dehydrogenase.* As shown in Fig. 5, animals injected with I^{131} in which treatment was delayed until the fifteenth day (Group II) or twentieth day (Group III) failed after 15 days of treatment to regain normal levels of succinic dehydrogenase activity. Two animals of Group II were

carried for 50 and 60 days respectively on replacement therapy without change in the level of suppression of succinic dehydrogenase activity.

It will be noted that the enzyme activity of the normal controls of these groups was lower by 1 unit than the values obtained in the first series of assays. We can explain this discrepancy only by the circumstance that for the replacement experiments a different strain of rats derived from a different dealer was used. The observation that different though related strains differ significantly in enzymatic and other biochemical characteristics has been repeatedly reported for various inbred strains of mice.

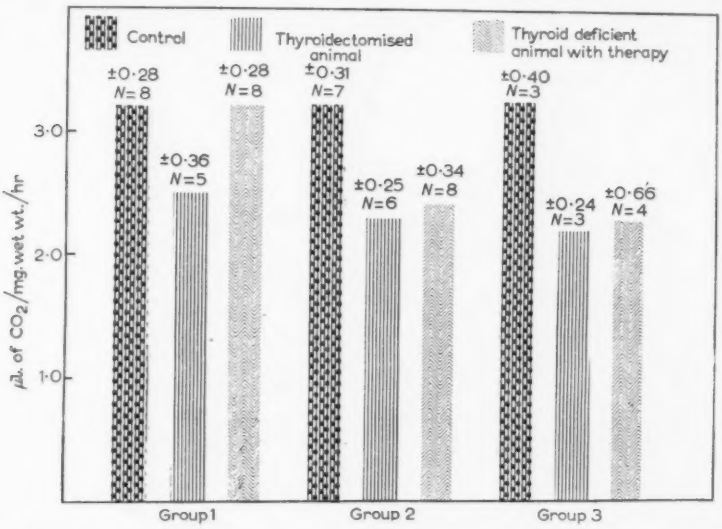


FIG. 5.—Effect of replacement therapy on succinic dehydrogenase activity of animals thyroidectomized at birth. In Group I hormone therapy was started at the tenth day and continued for 15 days; in Group II, therapy was started at the fifteenth day and continued for 15 days in six animals, for 50 days in one animal, and for 60 days in one animal; in Group III, therapy was started at the twentieth day and continued for 15 days. Over each bar, the height of which represents the mean, is placed the standard error of the mean, and N = the number of animals in the group. Note that animals for this series came from a different strain derived from a different dealer than those used for determinations represented in Fig. 1. The base values of the controls differ correspondingly by 1 unit.

DISCUSSION

1. *Normal pattern of enzyme change.* On the basis of assays of aldolase, succinic dehydrogenase, and cytochrome oxidase, it appears that development of the frontal cerebral cortex of the rat, like that of the guinea pig, is characterized by a period during which the activities of certain enzymes begin rapidly to increase to the adult level. These findings gain support from the observations that the activities of succinic dehydrogenase, cytochrome oxidase, ATPase (POTTER, SCHNEIDER, and LIEBL, 1945) and phosphoglucomutase (SHAPIRO and WERTHEIMER, 1943) in the whole brain of the rat begin to increase from the sixth to the tenth day after birth. As in the guinea-pig, cholinesterase begins to increase in activity considerably before the other enzymes. Further work is necessary to analyse the meaning of this finding with cholinesterase, since the cortex of the rat has been found to contain a considerable amount of non-specific cholinesterase (ORD and THOMPSON, 1952) which is present in cells other than nerve cells (KOELLE, 1954).

Morphological, functional, and biochemical evidence at hand strongly indicates that the nerve cells of the frontal cortex of the rat at or about the tenth postnatal day undergo the same kind of changes observed in the guinea-pig between the forty-first and forty-sixth days of gestation. A series of morphological changes, including changes in population density of cortical nerve cells, in Nissl substance, and in volume of cell nuclei have been observed (SUGITA, 1918) between the fifth and tenth days after birth. TILNEY (1933) first found evidence of cortical activity in the behaviour pattern of the rat around the fifteenth day. At 10 days, CRAIN (1952) observed an electroencephalogram basically similar to that of the adult. GREENGARD and MCILWAIN (1955) found with the rat's cerebral hemisphere that the *in vitro* metabolic response to electrical pulses increases at about the tenth day. The enzyme changes which we have found to occur shortly after the tenth day also fit the thesis that this is the time when cortical neuroblasts assume the characteristics of mature neurons.

2. *Pattern of enzyme change in hypothyroidism.* Three conclusions can be drawn from the observations: (1) The activities of enzymes of the developing cerebral cortex are affected to a different degree by hypothyroidism; some are unaffected (cytochrome oxidase and aldolase); others may be reduced to a greater (succinic dehydrogenase) or lesser degree (cholinesterase). (2) The effect on succinic dehydrogenase first becomes apparent after the tenth day, the critical time during postnatal development when the activity of the enzyme begins relatively sharply to rise to the adult level. (3) Cholinesterase, which, unlike the other enzymes studied here, rapidly increases in activity during the first 10 days of life, is significantly depressed in activity by the tenth day. With a sensitive enzyme of the immature cerebral cortex it appears that thyroid hormone is essential only for maintaining its normal rate of increase in activity. This conclusion must be tentative until other enzymes have been studied and until the period of hypothyroidism has been increased by producing thyroidectomy *in utero*.

It is uncertain whether absence of thyroid hormone leads to a decrease in rate of synthesis of succinic dehydrogenase and cholinesterase or whether it simply lowers the activity of a numerically normal population of enzyme molecules. DRABKIN (1950) found that the concentration of cytochrome *c* in several tissues is reduced in hypothyroidism and increased in hyperthyroidism. The activity of succinoxidase and cytochrome oxidase has also been shown to increase in hyperthyroidism (COHN and GERARD, 1937; TIPTON, 1950). Unlike cytochrome *c*, this effect is not necessarily the result of an increase in concentration of the enzymes, but may be due to an increase in their activities mediated by thyroid hormone, since it has been found that the activity of succinoxidase in an *in vitro* system is increased by addition of thyroxin (GEMMILL, 1952). Our results are tentatively most simply interpreted, however, by assuming that in absence of thyroid-hormone decrease in enzyme activity is a reflection of decrease in rate of synthesis. On this assumption, the early response of cholinesterase and the later response of succinic dehydrogenase are readily explained. If thyroid hormone is essential for normal rates of oxidative phosphorylation (MALEY and LARDY, 1953), the reduced amount of energy available in its absence might well lead to a differential effect on rate of synthesis of enzyme molecules.

3. *Effect of replacement therapy.* It has been pointed out that the tenth day is the time when succinic dehydrogenase begins rapidly to increase in its activity. Replacement therapy started at the tenth day and continued for 15 days led to a normal level of enzyme activity in animals given I^{131} at birth. Enzyme activity failed to respond

VOL
1
1956/

when therapy was delayed until the fifteenth or twentieth day. It is tempting to conclude with respect to succinic dehydrogenase that efficacy of treatment depends upon whether it is instituted before or after the critical period and that irreversible changes in absence of thyroid hormone occur at the critical period. This conclusion, however, is not justifiable without reservations. The amount of hormone used might not have been optimal under our experimental condition. Furthermore, improvement might have been obtained in unresponsive animals with longer periods of treatment or by taking more rigorous measures to guard against undernutrition. Finally, it may be argued that the positive response to therapy begun at the tenth day might be reproduced at a later age in the still immature animal if the interval between thyroidectomy and treatment were reduced to that in our Group I; or the treatment begun at 10 days might be ineffectual if the period of hyperthyroidism preceding treatment were increased by producing thyroidectomy *in utero*.

The electroencephalogram of the human foetus has been recorded at the seventh month of pregnancy (LINDSLEY, 1942). On the basis of the observations on guinea pig and rat, this may be taken as evidence that increase in activity of certain enzymes of the human cerebral cortex begins at or shortly before this time. Absence of thyroid hormone may consequently be regarded as capable of producing irreversible changes in succinic dehydrogenase activity well before birth of the human infant, if further experience substantiates our present evidence on the rat.

VOL. 1 56/57

SUMMARY

Succinic dehydrogenase, cytochrome oxidase, and aldolase of the frontal cerebral cortex of the rat begin rapidly to increase in activity at the tenth postnatal day. The most rapid increase in cholinesterase activity occurs before this time. Thyroidectomy at birth with I^{131} leads to a substantial decrease in activity of succinic dehydrogenase, a less marked depression of cholinesterase, and is without significant effect on the activities of cytochrome oxidase and aldolase. Hormone therapy in thyroidectomized animals when started at the tenth day led to a normal level of succinic dehydrogenase activity 15 days later, but was without effect when its initiation was delayed to the fifteenth or twentieth day.

Acknowledgement—We are obliged to Miss SOPHIE WYSIENSKA for technical assistance.

REFERENCES

AUGUSTINSSON K. B. (1948) *Acta Physiol. Scand.* (Suppl. 52) **15**, 1.
BARRNETT R. J. (1943) *Thesis*, Yale Univ. School of Medicine.
COHN R. A. and GERARD R. W. (1937) *J. Cell. Comp. Physiol.* **10**, 223.
COOPERSTEIN S. F. and LAZAROW A. (1951) *J. Biol. Chem.* **189**, 665.
CRAIN S. M. (1952) *Proc. Soc. Exp. Biol. and Med. N.Y.* **81**, 49.
DOUNCE A. L. and BEYER G. T. (1948) *J. Biol. Chem.* **173**, 159.
DRABKIN D. L. (1950) *J. Biol. Chem.* **182**, 335.
EAYRS J. T. (1953) *British J. Animal Behaviour* **1**, 144.
EAYRS J. T. (1954) *J. Anat., Lond.* **88**, 164.
EAYRS J. T. (1955) *Acta. Anat.* **25**, 160.
EAYRS J. T. and HORN G. (1955) *Anat. Rec.* **121**, 53.
EAYRS J. T. and LISHMAN W. A. (1955) *British J. Animal Behaviour* **3**, 17.
EAYRS J. T. and TAYLOR S. H. (1951) *J. Anat., Lond.* **85**, 350.
FLEXNER J. B. and FLEXNER L. B. (1948) *J. Cell. Comp. Physiol.* **31**, 311.

FLEXNER J. B., GREENBLATT C. L., COOPERBAND S. R., and FLEXNER L. B. (1956) *Amer. J. Anat.* **98,** 129.
FLEXNER L. B., BELKNAP E. L., and FLEXNER J. B. (1953) *J. Cell. Comp. Physiol.* **42,** 151.
FLEXNER L. B. and FLEXNER J. B. (1946) *J. Cell. Comp. Physiol.* **27,** 35.
GEMMILL C. L. (1952) *J. Clin. Endocr.* **12,** 1300.
GOLDBERG R. C. and CHAIKOFF I. L. (1949) *Endocrinology* **45,** 64.
GREENGARD P. and MCILWAIN H. (1955) Metabolic response to electrical pulses in mammalian cerebral tissues during development. In *Biochemistry of the Developing Nervous System* (Ed. H. Waelsch) Acad. Press., N. Y.
HORN G. (1955) *Anat. Rec.* **121,** 63.
KOELLE G. B. (1954) *J. Comp. Neurol.* **100,** 211.
LINDSLEY D. B. (1942) *Amer. J. Psychol.* **55,** 412.
LOWRY O. H., ROBERTS N. R., WU M., HIXON W. S., and CRAWFORD E. J. (1954) *J. Biol. Chem.* **207,** 19.
MALEY G. G. and LARDY H. A. (1953) *J. Biol. Chem.* **204,** 435.
ORD M. G. and THOMPSON R. H. S. (1952) *Biochem. J.* **51,** 245.
POTTER V. R., SCHNEIDER W. C., and LIEBL G. J. (1945) *Cancer Res.* **5,** 21.
QUASTEL J. H. and WHEATLEY A. H. M. (1938) *Biochem. J.* **32,** 936.
SHAPIRO B. and WERTHEIMER E. (1943) *Biochem. J.* **37,** 397.
SIBLEY J. A. and LEHNINGER A. L. (1949) *J. Biol. Chem.* **177,** 859.
SUGITA N. (1918) *J. Comp. Neurol.* **29,** 1.
TILNEY F. (1933) *Bull. Neur. Inst. N.Y.* **3,** 252.
TIPTON S. R. (1950) *Amer. J. Physiol.* **161,** 29.

VOL
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 289–300. Pergamon Press Ltd., London

AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—I

TURNOVER OF FREE AND PROTEIN BOUND LYSINE IN BRAIN AND OTHER ORGANS*

A. LAJTHA, S. FURST,† A. GERSTEIN, and H. WAELSCH
Department of Biochemistry, College of Physicians and Surgeons, Columbia University and the New York State Psychiatric Institute, New York, N.Y.

(*Received* 20 *December* 1956)

VOL. 1 6/57

SINCE the classical study of SCHOENHEIMER and associates (1939) on the metabolism of doubly-labelled L-leucine, a study which provided the first estimate of the half-life time of liver protein, the turnover rates of the proteins of many organs have been determined with the aid of labelled amino acids, in particular glycine and methionine (BORSOOK, 1950). Most of the estimates of protein turnover were based on the results of experiments of relatively long duration in which the appearance of the labelled amino acid in or the disappearance from the proteins was followed. However, in such experiments the average half-lifetime of mainly the relatively slowly metabolized protein will be measured; the turnover rates of the rapidly metabolized organ proteins would have to be calculated from the ascending portion of activity-time curves. During long-term experiments, proteins with a short half-lifetime lose part of their label, which is diluted out by the free amino acids of the body.

The earlier determinations of turnover values of proteins suffered also from the handicap of the lack of methods for the determination of the concentration and isotope content of the free amino acids from which the organ proteins are derived. Owing to this situation, the estimate of turnover rates had to be based on assumptions as to the nature and dynamics of the isotope pool from which the labelled protein constituents were drawn.

Despite these limitations, the investigations demonstrated clearly the relatively high turnover rates of the proteins of organs or tissues such as liver, kidney, or intestinal mucosa; on the other hand, the turnover rates of the proteins of organs such as brain or muscle were in doubt or considered very low.

The half-lifetimes of the proteins of the internal organs (spleen, heart, kidney, intestine, and testes) were estimated as close to that of liver proteins (7 days); those of skin, muscle, bone, lung, and brain were assumed to equal 158 days (SHEMIN and RITTENBERG, 1944; SPRINSON and RITTENBERG, 1949). In such an estimate any small organ with rapidly metabolized protein would, of course, not decisively change the average values derived for the half-lifetime of the slowly metabolized proteins.

* This work was supported by grants from the National Institute of Neurological Diseases and Blindness (Grants B-226 and B-557) of the National Institutes of Health, Public Health Service, United Cerebral Palsy, Scottish Rites Supreme Council, and by a contract between the Office of Naval Research and the Psychiatric Institute. Preliminary reports have appeared (LAJTHA and FURST, 1955; WAELSCH, 1956; LAJTHA *et al.*, 1957).

† Special Fellow, United States Public Health Service.

Not only indirect calculation but also direct determination (GREENBERG and WINNICK, 1948) appeared to indicate a very low relative uptake of isotopic amino acids by brain proteins and consequently a low rate of turnover. On the other hand, an incidental finding by FRIEDBERG *et al.* (1948) suggested that S^{35}-methionine administered intrathecally instead of intravenously was rapidly incorporated into the brain proteins. These observations pointed to the decisive role which the blood-brain barrier may play as an interfering factor in any investigations in which the turnover of brain proteins would be studied with experimental techniques with which the turnover of proteins of other organs may be investigated successfully. The importance of the blood-brain barrier as a regulating mechanism for the uptake of amino acids by and release from the brain was further impressed upon us by the observation that increased glutamic acid concentrations in the blood of mice and rats did not lead to a net increase in the concentration of this amino acid in the brain, while significant increases of glutamine were found in the brain after intravenous administration of the amide (SCHWERIN *et al.*, 1950). It appeared, therefore, that without a knowledge of the flux of an amino acid from the blood-stream into the brain and a consequent knowledge of the specific activity of the free test amino acid in the brain, no definite answer as to the rate of turnover of brain proteins could be expected.

We wish to report in this series of communications our studies of recent years in which, with C^{14}-lysine as the test amino acid, the turnover of brain proteins was investigated. Lysine was chosen, since it could be expected that, unlike the acidic glutamic acid, this basic amino acid might enter the brain from the blood-stream at a rate not too greatly influenced by the blood-brain barrier. This assumption was supported by the observation (KAMIN and HANDLER, 1951) that in constant-rate perfusion experiments with dogs the basic amino acids were found to enter the brain from the circulating blood. Furthermore, this amino acid is relatively stable metabolically and therefore offers distinct advantages over C^{14}-glycine and S^{35}-methionine. In short-time experiments, distribution of the label among other amino acids should not occur to a degree which would make it impossible to relate radioactivity directly to lysine occurring either free in the nonprotein fraction or bound in proteins. On the other hand, it had been reported (SCHWEET, 1955, 1956) that lysine may attach itself to proteins by linkage of its ε-amino group, probably to the γ-carboxyl group of glutamic acid.

Especially designed experiments showed that an insignificant amount of C^{14} appeared in amino acids other than lysine during experiments of short duration and also that the results were not distorted by lysine incorporated into other than α peptide linkage. After these facts had been ascertained, it was possible to determine the specific activity of lysine by determination of total radioactivity and of the lysine concentration by microbiological assay in either the nonprotein nitrogen fraction or protein hydrolysates.

Using this technique, the flux of lysine from the blood of mice into brain, liver, and muscle and from the nonprotein nitrogen fraction into the proteins of these organs was determined. The free lysine of the organ itself served as the basis of calculation of turnover rates of its proteins. From 2 to 60 min elapsed between the administration of the labelled lysine and the removal of the organs from the animal. Therefore, most of the values obtained were on the ascending portion of the activity-time curve. The flux of lysine into the nonprotein nitrogen fraction of the organs

VOL
1
1956/

from the blood as well as from this fraction into the organ proteins was determined in adult as well as in young mice at the onset of myelination.

While this work was in progress, two papers appeared in which the turnover of the proteins of liver and muscle was measured with the aid of C^{14} glycine (HENRIQUES *et al.*, 1955), and of the brain with S^{35} methionine (GAITONDE and RICHTER, 1956). In both sets of experiments the radioactivity of the free test amino acid in the non-protein fraction was taken as the basis for calculation. In the former series (HENRIQUES *et al.*, 1955) free glycine was isolated; in the latter (GAITONDE and RICHTER, 1956) an amount of S^{35} methionine was administered intrathecally which was supposed to dilute out the small amount of free unlabelled methionine. The authors felt justified in taking the specific activity of the administered methionine as the basis for calculation of turnover rates. Both groups of investigators performed experiments of short duration.

EXPERIMENTAL

Animal experiments. C_3H mice were used in all experiments. Adult animals (approx. 100 days old) were injected intravenously, and young animals (10 days old) intraperitoneally. Uniformly labelled lysine-C^{14} containing 13 mc/mM (Nuclear-Chicago) was used. In order to minimize any physiological disturbance, the volume injected was limited to less than 10% of the plasma volume, and the quantity of lysine introduced to less than 4% of the total free lysine of the plasma.

At the conclusion of the predetermined experimental interval (2 to 60 min), the animals were sacrificed by decapitation, the blood collected in a dry vessel containing a small amount of sodium citrate, and the plasma separated by centrifugation. The excised organs and the plasma were quickly frozen in dry ice and weighed.

Preparation of the protein fraction. All tissue and plasma samples were homogenized in 9 volumes of cold 5% trichloroacetic acid (TCA) and the suspension centrifuged. The residue was homogenized in 5% TCA, separated by centrifugation, and the procedure repeated. The combined protein free supernatant solutions were treated as described below. The residues were rehomogenized in 5% TCA and the suspension kept for 15 min in a water-bath at 90° for the removal of nucleic acids (SCHNEIDER, 1945). After one additional suspension and centrifugation in 5% TCA, the residues were washed successively three times with acetone, three times with ether, and dried, first at room temperature, and finally at 110°C for 15 min. This protein powder was used for determination of radioactivity. In addition, part of it was hydrolysed for the determination of its lysine content.

Hydrolysis of proteins. Samples containing about 15 mg of accurately weighed protein powder were hydrolyzed in 3 ml of glass-distilled 6 N-HCl in sealed tubes at 115°C for 18 hours. After quantitative transfer of the hydrolysate to small beakers, the samples were placed over KOH and P_2O_5 in a vacuum desiccator, which was kept at 50°C overnight. The resulting dry residue was taken up in distilled water, neutralised, and cleared of humin by centrifugation. Aliquots were used for the determination of lysine content by microbiological assay.

Preparation of nonprotein nitrogen (NPN) fraction. The combined supernatant fluids (see above) containing the NPN fractions in 5% TCA were prepared for microbiological assay and determination of radioactivity as follows: Trichloroacetic acid was removed by passing the solution through a column of Dowex-2 (X8-Cl form) resin at pH 6; the column was washed with five 4 ml portions of 0·02 N-HCl. About 0·5 ml wet resin (100–200 mesh) was used for each ml of 5% TCA, the usual volume being 25–50 ml resin in a column of 2-cm diameter. The combined effluent and washings (containing the NPN in dilute HCl) were brought to dryness *in vacuo* in a rotary flash evaporator. The residue was taken up in water, neutralized, made up to volume with H_2O, and aliquots were taken for microbiological assay and determination of radioactivity.

Determination of radioactivity. Radioactivity of all samples was determined in a gas-flow counter (Nuclear-Chicago Corp., Model C 110-A). A minimum of 10,000 counts was obtained for each sample, and all values were corrected for background and self-absorption. Aliquots of solutions containing the NPN were transferred to planchets. The solutions were taken to dryness under an infra-red lamp, with special precaution (rotation) to insure even plating. The radioactivity of the protein samples was determined by counting the dry protein powders at infinite thickness.

Isolation of lysine. In order to establish that the radioactivity of the proteins was due exclusively to their lysine content, aliquots of protein powders from different organs were hydrolysed and the lysine isolated from the hydrolysate. A modification of the method of WINTERS and KUNIN (1949) was used. After removal of HCl from the hydrolysate, the free amino acids were placed on a column of Amberlite IR C50 buffered at pH 7. Acidic and neutral amino acids as well as histidine are not absorbed under these conditions. Traces of these acids were removed by washing the column with 0·1 M phosphate buffer, pH 7. The adsorbed lysine and arginine were eluted with 5 ml portions of 1 N-HCl until the effluent was ninhydrin-negative. 2 g of wet resin (capacity 1·5 mEq/g wet resin) for each milliequivalent of lysine was used and the rate of flow regulated so as not to exceed 0·01 ml/min/g wet resin. The ratio of column diameter to length was about 1 : 50. The solution containing lysine and arginine was desalted by the method of WINZLER *et al.* (1952). Dowex-50 (X4 100–200 mesh in Cl form) was used in columns of 1 cm diameter. 1·3 g wet resin was used for each mEq salt contained in the solution. To the lysine-arginine solution hydrochloric acid was added to a concentration of 0·01 N- and the solution was run through the resin. After washing the column with 0·01 N-HCl and water in order to remove salts, the amino acids were eluted with 1 N-NH_4OH, and the effluent fluid brought to dryness *in vacuo* in a flash evaporator. The residue was dissolved in 0·001 N-NH_4OH, and this solution applied to a column of Amberlite IR 400. Columns of similar proportions as before were used andth e solutions run through at the same speed as in the case of the IR C50 resin. About 3 g wet IR 400 was used for each milliequivalent of lysine. Arginine was recovered by washing with additional solvent. Lysine was finally eluted with 1 N-HCl, the eluate was neutralized, and its lysine content was determined by microbiological assay and its radioactivity as described above. Similarly, the radioactivity of the arginine fraction and of the fraction containing the rest of the amino acids was determined. In a typical experiment, 4440 counts/min were found in the lysine fraction, 33 counts/min in the arginine fraction (0·7%), and 144 counts/min in the fraction containing the rest of the amino acids (3·2%).

Microbiological assay. The procedure of HENDERSON and SNELL (1948) was followed, *Leuconostoc mesenteroides* P-60 being used as the test organism. The final volume of each sample was adjusted to 4 ml and, after incubation, the tubes were read in a Bausch and Lomb "Spectronic 20" spectrophotometer at 525 mμ. In each experiment several standard lysine concentrations were determined simultaneously.

Blood determination. In order to ascertain the extent of the contribution of residual blood to the radioactivity of the organs, the blood content of each organ studied was determined. A modification of the method of GORDON and NURNBERGER (1955; 1956) was used, which is based on a determination of CO-haemoglobin. The tissues were homogenized in water containing 0·4% saponin and the homogenate centrifuged at 12,000 × g. The supernatant fluids were divided into two aliquots, one of which was gassed with CO and the other with air. Gassing was followed by centrifugation at 100,000 × g for 30 min. The optical densities of the samples were read at 562 and 600 mμ (Beckman spectrophotometer) and related to a standard curve prepared by subjecting dilutions of whole blood of the same animal to an identical procedure (Table 1).

TABLE 1.—DETERMINATION OF BLOOD IN MOUSE ORGANS

	Per cent blood				
	Adult			Young	
Brain	0·8	0·4	0·9	0·6	1·0
Liver	2·4	2·0	3·2	3·2	4·0
Muscle	0·6	0·4	0·9	0·8	0·9

Determination of the lysine other than bound by α-peptide linkage. Any isotopic lysine present in protein samples either adsorbed or bound by the ε-amino group would contain a free α-amino and carboxyl group and would, under ninhydrin treatment, liberate the carboxyl as carbon dioxide. The radioactivity of the carbon dioxide sample relative to the whole amount of isotope incorporated could serve as a measure of lysine bound other than by α-peptide linkage. Therefore, the distribution

of C^{14} in the carboxyl group and the rest of the lysine molecule was estimated by determining the radioactivity of the barium carbonate obtained from carbon dioxide upon treatment of the amino acid with ninhydrin, according to the method of VAN SLYKE *et al.* (1941). A considerable amount of this C^{14}-lysine was then injected into mice, and samples of brain and liver protein were subjected to the ninhydrin procedure and the radioactivity of the liberated carbon dioxide was measured (Table 2). A negligible fraction of the total counts was found in the carbon dioxide in some of the experiments. The results show that the radioactivity found in the protein samples may be referred to lysine bound in α-peptide linkage.

TABLE 2.—DETERMINATION OF C^{14}-LYSINE NOT BOUND BY α-PEPTIDE LINKAGE IN PROTEINS

Organ	Total counts found in protein sample	Calculated counts in carboxyl C*	Counts found in carbon dioxide (ninhydrin)	Per cent of total carboxyl counts in carbon dioxide
Plasma	24,730	6,730	0	0
Brain	6,582	1,790	2·5	0·14
Brain II	1,486	404	0·5	0·12
Liver	32,300	8,790	0	0
Muscle	4,320	1,175	0	0

* The lysine used contained, per microgram, 71,230 total counts and 19,380 counts in the carboxyl carbon.

Calculation of the flux of lysine. The flux of lysine from the circulating blood into NPN of the various organs was calculated as γ lysine transported per minute per 1 g fresh tissue and the flux from the free organ lysine into the protein bound lysine as γ lysine per milligram dry protein per minute according to the formula (REINER, 1953):

$$F = C\frac{\frac{dSb}{dt}}{Sa - Sb}$$

F denotes flux of lysine in the above units, *Sa* the specific activity of the precursor, *Sb* the specific activity of the product, and *C* the concentration of *Sb*. Specific activity equals counts per γ lysine per minute. In the case of the flux of lysine into the free amino acid pool, *Sa* is the specific activity of plasma-free lysine and *Sb* of the free lysine of the organ; in the calculation of lysine flux into proteins, *Sa* denotes the specific activity of the free lysine of the organ and *Sb* the specific activity of the lysine bound in the organ protein.

dSb/*dt* was estimated graphically from specific activity/time curves.

For the purpose of easier visualization, the half-lifetime ($t_{\frac{1}{2}}$) of the free lysine in the organ and of the protein lysine was calculated from the flux values:

$$\frac{C \times \ln 2}{F} = t_{\frac{1}{2}} \text{ in minutes (free organ lysine).}$$

$$\frac{C \times \ln 2}{F \times 1440} = t_{\frac{1}{2}} \text{ in days (protein).}$$

This calculation assumes that we are dealing with first-order reactions. For discussion of this problem see REINER (1953).

RESULTS

Exchange of free lysine between blood and brain. Lysine-C^{14} administered intravenously or intraperitoneally disappears rapidly from the circulating blood and enters liver, brain, muscle, and kidney in both young and adult mice, as shown by the data in Table 3 and Fig. 1. While the highest rate of entry is into liver, the amino acid is also taken up by the brain at a high rate. Approximately 30 min after injection,

the specific activity of the free lysine of the brain equals that of the plasma. Brain and muscle exchange their lysine with that of the plasma at approximately equal rates.

TABLE 3.—SPECIFIC ACTIVITY OF FREE LYSINE OF MOUSE ORGANS AFTER ADMINISTRATION OF C^{14}-LYSINE

Time after administration	Counts/γ lysine/min			
	Plasma	Brain	Liver	Muscle
2	2,570	280	1,640	330
5	1,200	420	1,880	310
10	870	430	1,310	430
20	280	360	510	400
30	170	250	700	240
45	120	240	230	300
60	76	250	230	120

Lysine content of organs/g fresh tissue: plasma 63 γ; brain 37 γ; liver 92 γ; muscle 160 γ.

The changes with time of the specific activities of the free lysine of plasma and brain serve for the calculations of the flux of the free lysine from the blood into the brain. The equations are based on the assumption that the free amino acids of the plasma are the precursors of the free amino acids in the organ, and that both plasma

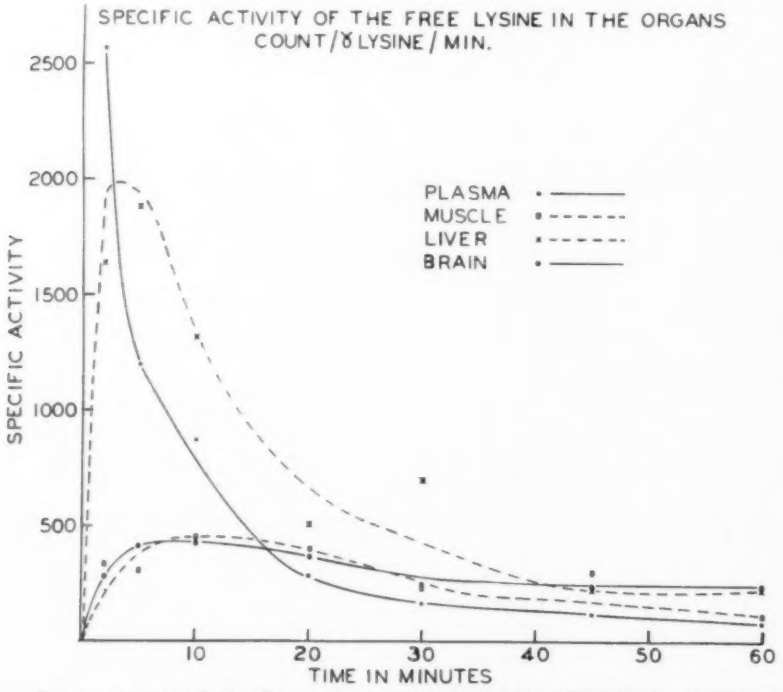


FIG. 1.—Specific activity of free lysine in mouse organs after administration of C^{14}-lysine

and organ pools are uniform. The results of calculations of the flux of the free lysine pool of the brain are given in Table 4, which shows the amount of lysine entering the pool from the plasma per unit of time. During the course of short-term experiments

TABLE 4.—FLUX (F) OF FREE LYSINE FROM BLOOD INTO BRAIN AND LIVER; HALF-LIFETIME ($t_{\frac{1}{2}}$) OF FREE LYSINE OF BRAIN AND LIVER (ADULT AND YOUNG MICE)

Duration of experiments in minutes	Brain				Liver			
	Adult		Young		Adult		Young	
	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$
2	2·3	11			29	2·2		
3	0·7	43						
5	1·3	24	1·2; 1·1	28; 29	10	5·8		
10			1·5; 1·1	21; 29	20	3·2	24; 63	2·2; 0·8
20	5·2	5·0			6·9	9·2	8·5	6·0
30	1·5	17			6·3	10·0		
45							11; 8·4	5·0; 6·5

$F = \gamma$ lysine/g fresh tissue/min; $t_{\frac{1}{2}}$ expressed in minutes.

the specific activity of the free lysine of the plasma changes very rapidly. The calculations, therefore, are approximations, a fact borne out by the variation in values given in Table 4. The data are, however, of the same order of magnitude, and are apparently lower for brain than for liver, while the difference between the value for brain of adult and young mice is not striking.

If, in addition to flux, the free lysine content of an organ is known, the turnover rate of the free lysine pool of the organ may be computed. Table 4 also gives the half-lifetimes of the free lysine pools of brain and liver as arrived at in this manner. Turnover appears to be very rapid, the largest value found for the half-lifetime of the free lysine of the brain being about 45 min, and that of the liver about 10 min.

Rate of synthesis of brain proteins. The labelled lysine of the free amino acid pool of the organs of adult and young mice is incorporated into cell proteins at a measurable rate, as is indicated in Table 5 and Fig. 2. Not only are the liver proteins

TABLE 5.—SPECIFIC ACTIVITIES OF ADULT MOUSE ORGAN PROTEINS AFTER ADMINISTRATION OF C^{14}-LYSINE

Time in minutes	Counts/γ lysine/min			
	Plasma	Brain	Liver	Muscle
2	0·06	0·08	1·0	0·04
5	0·15	0·23	4·5	0·12
10	0·06	0·51	5·6	0·18
20	2·0	0·75	8·1	0·29
30	5·0	0·99	7·3	0·46
45	8·2	1·3	6·9	0·60
60	6·8	1·4	6·1	0·68

Three 100-day-old mice were pooled for each time-point.

labelled rapidly, but the brain proteins also show significant incorporation of lysine even in experiments of short duration.

The formulae employed for the computation of the flux of lysine from blood to brain and liver may also be used for the calculation of the flux of the free lysine of an organ into its proteins, the underlying assumption being that the amino acids of the nonprotein nitrogen fraction are the precursors of organ proteins. Thus, if the

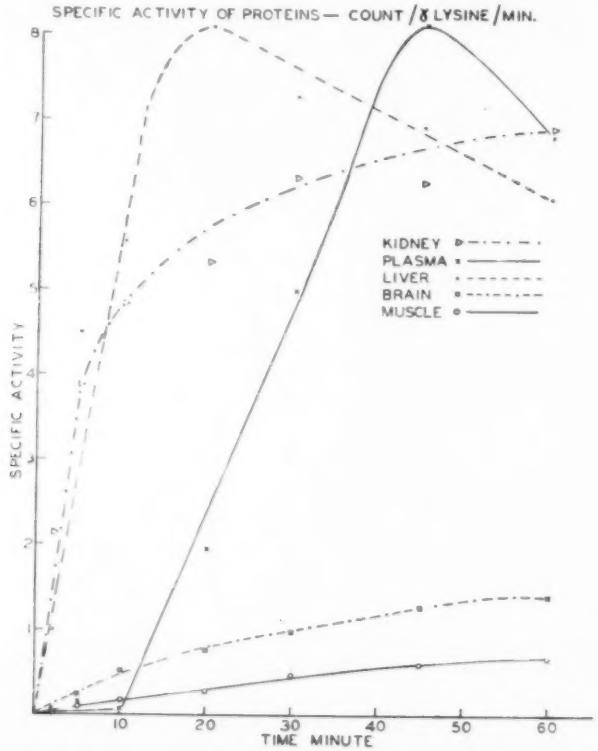


FIG. 2.—Specific activity of protein lysine of mouse organs after administration of C^{14}-lysine

changes with time of specific activities of both the free lysine and the protein-bound lysine of the organ are known, the flux of lysine into the proteins as well as the half-lifetime of the proteins may be ascertained (Table 6). It is seen that the protein half-lifetimes calculated for each organ are not constant, but show an apparent increase with each succeedingly longer time-interval studied. This is explicable on the basis of the fact that all the proteins of an organ do not turn over at a uniform rate. In short-term experiments, those proteins with a rapid turnover rate influence the result most heavily, while, in experiments of longer duration, proteins with a lower turnover rate affect the result more markedly. The implications of this finding are dealt with in further detail in the discussion section below.

DISCUSSION

The use of lysine as an indicator of protein metabolism offers several distinct advantages over the use of amino acids such as glycine or methionine. Lysine is a

ubiquitous constituent of proteins and its conversion into glutamic acid is relatively slow. Thus, any radioactivity found in protein in short-time experiments, following the injection of C^{14}-lysine, can be attributed to the incorporation of this amino acid *per se*, a fact confirmed by control experiments reported above.

TABLE 6.—HALF-LIFETIME (t) OF PROTEINS OF MOUSE ORGANS FLUX (F) OF LYSINE FROM NPN INTO PROTEIN

Time after administration (min)	Brain				Liver				Muscle			
	Adult		Young		Adult		Young		Adult		Young	
	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F	$t_{\frac{1}{2}}$	F
2	2·8	11·3			0·90	44·3			3·5	14·2		
5	3·5	9·1	3·4	9·6	1·25	32·0	1·73	23·3	7·6	6·5	2·4	20·1
10	5·5	5·8	2·2	14·9	2·57	13·5	1·10	36·7	7·6	6·5	1·7	28·3
20	6·2	5·1	3·0	10·9			2·68	15·1	10·4	4·8	2·5	19·2
30	6·9	4·6							13·2	3·8		
45	10·4	3·1	2·4	13·7					16·6	3·0	2·3	20·9
60	15·2	2·1							23·6	2·1		

$t_{\frac{1}{2}}$ in days; $F = \gamma$ lysine/mg protein/min.

Another advantage of the use of C^{14}-lysine in studies of protein metabolism lies in the fact that this amino acid occurs, as far as is known on the basis of direct analysis, in at least as high concentrations in the proteins of the parenchymatous tissue as in those of the connective tissue. Contrary to this, glycine occurs in very high concentrations in the protein of the connective tissue, and in any study of turnover rate carried out with glycine one would have to expect a considerable dilution with glycine from proteins which are suspected to have a slow rate of metabolism.

Lysine is particularly suitable for studying the protein metabolism of the brain tissue. As shown in this report, free lysine of the brain is rapidly exchanged with that of the circulating blood. It is of interest to compare the short half-lifetime of free lysine of the brain with that of brain potassium, which, on the basis of recent data (KATZMAN and LEIDERMAN, 1953), may be calculated at 24 hours. The measurements of the movement of C^{14}-lysine demonstrates that the free lysine of the brain is replaced by plasma lysine within 1 hour. This does not mean that the blood-brain barrier permits the unrestricted entrance of lysine into the brain. In fact, experiments to be reported show a negligible net uptake of lysine by the brain of the adult animal even when the blood concentration is increased considerably.

The values for specific activity of free lysine in the different mouse organs recorded in Table 3 are corrected for any contamination by blood. These corrections are based on the average blood content found in the organs of the animals (Table 1.) The question arises as to what proportion the C^{14}-lysine found in an organ may be located in the extracellular phase and may not have entered the intracellular compartment. This question cannot be answered for the brain since the blood-brain barrier is assumed to be located between the circulating blood and any extracellular space of this organ (LAJTHA, 1956). It is apparent that most of the lysine (70%) has already entered the intracellular compartment of the liver within 2 min after the

administration of C^{14}-lysine. On the other hand, in the case of muscle all the C^{14}-lysine, in the 2 min experiment, may still be located in the extracellular space (which is assumed to comprise 16% of the organ), while in the 10 min experiment 70% of the administered lysine may be calculated to have entered the intracellular compartment.

These estimates are of a certain interest, since it may be assumed that the synthesis, particularly of the rapidly metabolized proteins, occurs in the intracellular space. It is, therefore, conceivable that the values of flux from the free into the protein-bound lysine are actually larger than calculated. A similar uncertainty is introduced by lack of knowledge concerning the lysine distribution between the extra- and intracellular compartments of the tissues. Apart from these limitations, the data show clearly that in all organs studied the flux of free lysine into protein lysine diminishes with increasing time-intervals between the administration of C^{14}-lysine and sacrifice of the adult animal. Concomitantly, the half-lifetime of the proteins of the respective organs increases.

The speed at which free lysine is incorporated into brain proteins indicates that the turnover rate of these proteins is not too dissimilar from those found for the proteins of other organs such as liver and muscle.

The synthesis of protein in a given organ or tissue is attributable to two separate and distinct processes. Formation of new protein accompanies cell regeneration, and would simulate protein turnover in tissues in which continuous death and replacement of cells occur. On the other hand, proteins as intracellular constituents may be synthesized and degraded within the cell, and their metabolism is indicative of a true dynamic state of the cellular proteins. The two processes, of course, can and do occur simultaneously. The rapid rate of protein synthesis in brain must be attributed primarily to turnover of intracellular protein, rather than to the formation of new cells. As is well known, neuronal cells are not capable of regeneration, while the rate of mitosis of glial elements of brain is not nearly rapid enough to account for the rates of incorporation of lysine found in our experiments.

The finding that calculated turnover time becomes progressively longer as the duration of the experiment is increased is interpreted as evidence of the metabolic inhomogeneity of organ proteins. Formulae employed in the calculation of turnover rates assume a homogeneous system, in which all component proteins are turning over at the same rate. In such a system, the determination of turnover rate would be unaffected by the changes in the duration of the experiment. The converse is true for systems containing proteins with dissimilar metabolic rates. Here experiments of short duration will reflect primarily the activity of the fast fractions, resulting in a calculated short turnover time—superimposed on the turnover of slowly metabolized proteins. Experiments of long duration do not fully reflect the extent of activity of fast fractions, since these may well have been degraded and resynthesized during the time-interval studied. It follows that, under the latter conditions, the activity of the slower fractions will weight the result more heavily, giving a long calculated half-lifetime which would appear to be relatively constant. The results will still be weighted in favoru of the slowly metabolized proteins if an amino acid such as glycine, which occurs in particularly high concentrations in this protein fraction, is selected as the tracer, while a test amino acid such as lysine would probably favour the rapidly metabolized proteins.

VOL.
1
1956/5

The inhomogeneity of the tissue proteins has been commented on by several investigators, as it concerns the determination of turnover rates (FRIEDBERG *et al.*, 1948; GREENBERG and WINNICK, 1948; TARVER and SCHMIDT, 1942), although the fact has not been demonstrated in rate studies up to now. The spectrum of turnover rates of proteins found in the analysis of an organ is the composite of the varying rate of different cellular proteins and of the proteins of different tissues of an organ such as parenchymatous and connective tissues. Depending on their metabolic activity, the proteins of different tissue origin may coincide with each other or extend the spectrum of turnover rates. The question is of particular significance for the brain, where different areas vary considerably not only in the types and numbers of cells but also in specific structures such as grey and white matter.

It is of interest to note that in the young animal the protein fractions with slow turnover rates appear to be missing, while the fastest fractions show equal rates in both young and adult animals (Table 6). This finding is reminiscent of the observation (WAELSCH *et al.*, 1941) that during the period of myelination, during which the content of fatty acids in the brain doubles, the rate of deuterium incorporated with fatty acids did not show any indication of the increased synthetic activity. It appears that in the case of proteins too the turnover is rapid enough to encompass synthesis of additional proteins without producing an observable increase in rate.

In the experiments of GAITONDE and RICHTER (1956) mentioned above, in which S^{35}-methionine was injected intrathecally into rats, a half-lifetime of $13{\cdot}4 \pm 4{\cdot}1$ days was found in short-term experiments. The half-lifetime found did not vary with duration of the experiment. The discrepancy between their results and ours is probably to be accounted for in terms of the intravenous vs. the intrathecal route of administration. In this connection, it is significant that, following intracisternal injection of radiophosphorus, the degree of radioactivity was found to diminish progressively with distance from the ventricular walls (BAKAY, 1954). It is likely that in these short-time experiments in which methionine was administered intrathecally, the amino acid was not evenly mixed within the interstitial fluid and that a large part of the rapidly metabolized protein had in fact incorporated methionine of a considerably lower specific activity than the S^{35}-methionine administered.

In our investigation, as in most previous ones dealing with this subject, the rate of incorporation of *one* labelled amino acid into proteins was taken as a measure of the rate of protein turnover; in the strict sense, however, it permits conclusions only as to the turnover of the lysine of the proteins. However, recent studies (SIMPSON and VELICK, 1954) on the incorporation of several labelled amino acids into proteins which were subsequently isolated in pure form suggest that the renewal of proteins takes place throughout the whole molecule. While they do appear to demonstrate the precursor-product relationship for free amino acid and proteins, the existence of transient intermediates in protein synthesis is at present not excluded.

SUMMARY

Uniformly labelled radiolysine was injected into young and adult mice, and the specific activity (count/γ lysine) of the free and protein-bound lysine in plasma, brain, liver, and muscle was determined as function of time in short-time experiments.

From the specific activity-time curves, the flux and the half-lifetime of free lysine

and protein-bound lysine was calculated. The half-lifetime of free lysine of liver was found to be less than 10 min, and of brain less than 40 min.

The calculated half-lifetime of organ proteins varied with the duration of the experiments, being shorter in short-term experiments than in experiments of longer duration. This was interpreted to indicate the inhomogeneity of the proteins of an organ from the standpoint of turnover time. The following values for the half-lifetime of the most rapidly metabolized proteins were calculated: brain, 2·2 days; liver: 0·9 days; muscle, 1·7 days.

The half-lifetime of the free lysine of the brain in young animals was not shorter than in the adult nor was the half-lifetime of the most rapidly metabolized proteins. On the other hand, the slowly metabolized protein fractions were missing in the brain of the young animal.

These findings are discussed from the point of view of the blood-brain barrier and protein metabolism in general.

REFERENCES

BAKAY L. (1954) *Arch. Neurol. Psych.* **71,** 673.
BORSOOK H. (1950) *Physiol. Rev.* **30,** 206.
FRIEDBERG F., TARVER H., and GREENBERG D. M. (1948) *J. biol. Chem.* **173,** 355.
GAITONDE M. N. and RICHTER D. (1956) *Proc. Roy. Soc.* **B 145,** 83.
GORDON M. W. and NURNBERGER J. I. (1955) *J. Histochem. Cytochem.* **3,** 130; (1956) **4,** 84.
GREENBERG D. M. and WINNICK T. (1948) *J. biol. Chem.* **173,** 199.
HENDERSON L. M. and SNELL E. E. (1948) *J. biol. Chem.* **172,** 15.
HENRIQUES O. B., HENRIQUES T. B., and NEUBERGER A. (1955) *Biochem. J.* **60,** 409.
KAMIN H. and HANDLER P. (1951) *J. biol. Chem.* **188,** 193.
KATZMAN R. and LEIDERMAN H. P. (1953) *Amer. J. Physiol.* **175,** 263.
LAJTHA A. and FURST S. S. (1955) *Second Symposium on Neurometabolism* Chicago. In press.
LAJTHA A., FURST S. S., and WAELSCH H. (1957) *Experientia* In press.
LAJTHA A. (1957) *J. Neurochem.* In press.
REINER J. M. (1953) *Arch. Biochem. Biophys.* **46,** 53.
SCHNEIDER W. E. (1945) *J. biol. Chem.* **161,** 293.
SCHOENHEIMER R., RATNER S., and RITTENBERG D. (1939) *J. biol. Chem.* **130,** 703.
SCHWEET R. (1955) *Federation Proc.* **14,** 277; (1956) **15,** 350.
SCHWERIN P., BESSMAN S. P., and WAELSCH H. (1950) *J. biol. Chem.* **184,** 37.
SHEMIN D. and RITTENBERG D. (1944) *J. biol. Chem.* **153,** 401.
SIMPSON M. V. and VELICK S. F. (1954) *J. biol. Chem.* **208,** 61.
VAN SLYKE D. D., DILLON R. T., MACFADYEN D. A., and HAMILTON P. (1941) *J. biol. Chem.* **141,** 627.
SPRINSON D. and RITTENBERG D. (1949) *J. biol. Chem.* **180,** 715.
TARVER H. and SCHMIDT C. L. A. (1942) *J. biol. Chem.* **146,** 69.
WAELSCH H., SPERRY W. M., and STOYANOFF V. A. (1941) *J. biol. Chem.* **140,** 885.
WAELSCH H. (1957) in *Metabolism of the Nervous System* (Ed. D. RICHTER) Proceedings of the Second International Neurochemical Symposium, Pergamon Press, London. In press.

VOL.
1
1956/5

PRELIMINARY NOTE

The proteins of the brain in patients with lowered convulsion threshold

(*Received* 12 *February* 1957)

KAPS (1954) reported an increased albumin content in the soluble protein fraction of brain tissue taken from patients with cerebral tumours.

In a quantitative study of the soluble proteins of the brain by the Tiselius and paper electrophoretic methods, we have found that the electrophoretic patterns in four healthy subjects who died of perforating wounds resembled those of dog and ox brain (KIYOTA and SATO, 1956). Similar electrophoretic patterns, which were apparently normal, were obtained with brain samples from one manic and seven schizophrenic patients. The brain samples of four of the schizophrenics were obtained by biopsy: the other brain samples were taken within 6 hours after death. There was no evident age-difference in the soluble protein fraction in the above twelve cases, and also little regional difference in the brains examined. (For the ages of the patients and controls, and the regions of brains examined, see Table 1.)

TABLE 1.

	Controls (4 healthy, 7 schizophrenics, 1 mania)		Brain tumour			Epilepsy	
Ages	26, 28, 32, 33, 36, 37, 39, 40, 48, 49, 51, 58.		28, 44, 48, 51, 54, 57, 59, 62			24, 28, 30, 31, 34.	
Regions of brain	Front., pariet., temp. and occipit. lobe		Part distant from tumour		Part adjacent to tumour	Frontal lobe	
	Grey matter	White matter	Grey matter	White matter	White matter	Grey matter	White matter
No. of cases	12	11	8	8	6	5	5
Cases with large differences	0	0	5	5	6	3	4

A further study was made of the soluble protein fraction of brain tissue taken from eight patients with cerebral tumours, one with collagen disease, and five with idiopathic epilepsy. The brain samples from the patients with cerebral tumours and collagen disease, which were taken within 6 hours after death, showed oedema; the samples from the patients with epilepsy, which were obtained by biopsy, showed no macroscopic or histological abnormality.

Grey matter from the brains of patients with epilepsy, cerebral tumours, and collagen disease showed a higher albumin content than normal: this was found on examination by both electrophoretic methods. The *white matter* from the brains of epileptics resembled that adjacent to a tumour in showing a relatively high α-globulin content, when tested by the Tiselius method. The white matter from epileptics also resembled that from patients with cerebral tumours in showing a high albumin content by the paper electrophoretic method. This characteristic of the brain of epileptics was found in the grey matter in three out of five cases, and in the white matter in four out of five cases.

It appeared that the patients showing an increased susceptibility to convulsions (cerebral tumour, collagen disease, and epilepsy) had in common a relatively high albumin or α-globulin content in the soluble proteins of the brain. It has been shown elsewhere that a high albumin and α-globulin content

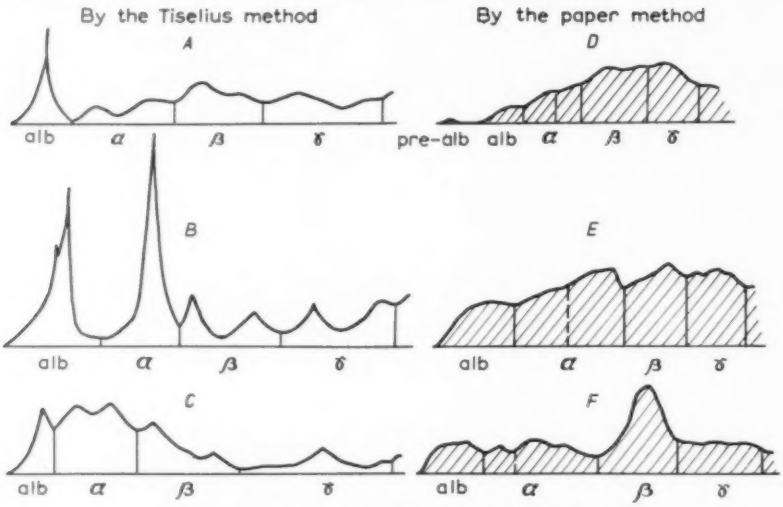


FIG. 1.—Electrophoretic pattern of extract of white matter:
(A), (D) normal
(B) patient with a tumour of frontal lobe (part adjacent to tumour)
(E) patient with metastatic neoplasm of frontal lobe (part adjacent to tumour)
(C), (F) patient with epilepsy (frontal lobe).
Each fraction of soluble protein in brain tissue is described provisionally as alb, α, β-, and γ-g lb, comparing them to the mobilities of serum protein fractions.
The "pre-albumin" fraction was found in about two-thirds of all samples, whether normal or abnormal.

is characteristic of the immature brain, and that these proteins play an important part in determining the water content of the tissue (KIYOTA, 1956). It is concluded that the brains of patients with idiopathic epilepsy may contain immature structural components with hydrophilic properties which make them especially liable to oedema and hence to convulsions.

K. KIYOTA

Department of Neuropsychiatry
Faculty of Medicine
Kumamoto University
Japan

REFERENCES

KAPS G. (1954) *Arch. Psychiat. Neurol.* **192,** 115.
KIYOTA K. and SATO N. (1956) *The Physico-Chem. Biol.* **3,** No. 2.
KIYOTA K. (1956) *Psychiat. et Neurol. Japonica* **58,** No. 3. 191.

VOL
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 303-310, Pergamon Press Ltd., London.

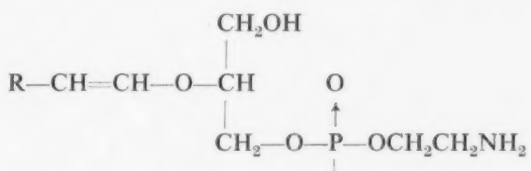
THE STRUCTURE OF PLASMALOGENS—III

THE NATURE AND SIGNIFICANCE OF THE ALDEHYDOGENIC LINKAGE

MAURICE M. RAPPORT and ROBERT E. FRANZL*

(*Received* 21 *February* 1957)

PLASMALOGENS are phosphoglycerides which liberate higher fatty aldehydes on hydrolysis under acidic conditions. They are widely distributed in animal tissues but are present in highest concentration in the myelin of brain and nerve, and in muscle, in particular, heart and skeletal muscle. In the first two papers of this series (RAPPORT and FRANZL, 1957; RAPPORT, LERNER, ALONZO, and FRANZL, 1957), the history of these substances was summarized, from their discovery in 1924 by FEULGEN and VOIT based on histochemical staining with fuchsin-sulphurous acid, to their recent status as lipids with incompletely established structure. Evidence was presented relating both to the position of attachment of the aldehydogenic chain to the glycerol residue and to the nature of the aldehydogenic linkage. In crystalline ethanolamine plasmalogen, obtained after alkaline hydrolysis and designated 'Acetal Phospholipide' by earlier workers (FEULGEN and BERSIN, 1939; THANNHAUSER, BONCODDO, and SCHMIDT, 1951), this linkage was shown to be an α,β-unsaturated ether (formula I) from the following evidence. The molecule contained a single unsaturated bond and,



I R = fatty chain

with acid, yielded one mole of higher fatty aldehyde per gram atom of phosphorus. After addition of hydrogen, an acid stable (saturated ether) linkage was produced with concomitant disappearance of aldehydogenic properties.

It is the purpose of this paper to present independent evidence confirming this assignment of structure of the aldehydogenic linkage as an α,β-unsaturated ether, and to show that it is present in other plasmalogens: in native as well as in lyso forms (obtained after alkaline or enzymic hydrolysis), in choline as well as in ethanolamine derivatives, and in those extracted from brain as well as from muscle.

* With the technical assistance of BERNARD LERNER and NICHOLAS ALONZO Division of Laboratories and Research, New York State Department of Health, and the Division of Experimental Pathology, Sloan-Kettering Institute for Cancer Research; and Sloan-Kettering Division, Cornell University Medical College, New York, New York.†

† Mailing address: Sloan-Kettering Institute, 410 East 68th Street, New York 21, New York.

α,β-Unsaturated ethers are unusually reactive organic structures and participate with ease in addition, hydrolysis, and polymerization reactions. Since plasmalogens are the first natural products in which such a structural arrangement has been found, we shall discuss briefly some of the relevant organic chemistry and its possible biochemical significance.

METHODS AND MATERIALS

(a) *Native plasmalogens.* A kephalin fraction containing a mole of ethanolamine per gram atom of phosphorus and enriched with respect to plasmalogen content was prepared by chromatographic fractionation of bovine muscle phospholipids on silicic acid as previously described (RAPPORT, LERNER, and ALONZO, 1954).

Choline plasmalogen is beef heart lecithin prepared according to PANGBORN (1951), which was shown to contain about 60 per cent plasmalogen and 40 per cent phosphatidyl choline (RAPPORT and ALONZO, 1955b).

(b) *Lysoplasmalogens.* Lysophosphatidal ethanolamine was prepared by alkaline hydrolysis from the phosphatidal ethanolamine-enriched kephalin fraction as previously described (RAPPORT *et al.*, 1957). In more recent studies it was possible to crystallize the substance directly after chromatography on silicic acid at 5°C without resorting to the use of Amberlite exchange resin. The column apparently effects the removal of cations. Lysophosphatidal ethanolamine is eluted with 50% ethanol in hexane in the fractionation method we employ (RAPPORT and ALONZO, 1956). Preparations of this compound from brain were obtained following the directions of THANNHAUSER, BONCODDO, and SCHMIDT (1951), substituting chromatography on silicic acid at 5°C for their methods after neutralization of the alkaline hydrolysate. The product was recrystallized from ethanol. Lysophosphatidal choline was obtained by alkaline hydrolysis of beef heart lecithin followed by chromatography. Details will be described in a later publication.

Lysophosphatidal choline mixed with lysophosphatidyl choline was obtained by enzymic hydrolysis of beef heart lecithin as previously described (RAPPORT and FRANZL, 1957).

(c) *Other phospholipids.* Egg phosphatidyl choline was prepared according to PANGBORN (1951). Vegetable phosphatidyl choline was purified chromatographically on aluminum oxide (HANAHAN, TURNER, and JAYKO, 1951).

(d) *Model compounds*

Oleic acid. Commercial specimen, redistilled under nitrogen, iodine no. 92.

Dihydropyran. Eastman Kodak Co. Redistilled, b.p. 85°C at atm. pressure.

Vinyl 2-ethyl hexyl ether. Carbide and Carbon Chemicals Co. Redistilled, b.p. 62°C at 12 mm.

Vinyl iso*butyl ether.* Carbide and Carbon Chemicals Co. Redistilled, b.p. 83°C at atm. pressure.

Palmityl dimethylacetal and myristyl glyceryl acetal (1:2-0-tetradecylideneglycerol) were synthetic preparations.

(e) *Hydrogenation.* Catalytic hydrogenation was carried out in Warburg vessels without a centre well according to the method of MEAD and HOWTON (1950), modified as previously described (RAPPORT *et al.*,1957).

(f) *Addition of bromine.* This was determined by the method of TRAPPE (1938) using methanolic bromine, instead of that of YASUDA (1931) in which pyridine sulphate dibromide is used in an acidic solvent. This was to minimize the possibility of interference due to the rupture of acid sensitive linkages. However, significant differences were not observed between the quantity of bromine added under the conditions described by TRAPPE and those employed in the modified YASUDA procedure used in this laboratory.

(g) *Addition of iodine.* SIGGIA and EDSBERG (1948) described a method for the quantitative determination of vinyl alkyl ethers based on the addition of iodine in methanolic solution. The method was reported to be specific in the presence of alcohols, acetaldehyde, acetals, acetylene, and water. We have found it to be specific for α,β-unsaturated ethers in the presence of ordinary olefinic unsaturation (Table 1). For non-volatile materials we have modified the SIGGIA-EDSBERG procedure as follows. To 0·01 mmoles of substance dissolved or suspended in 5 ml of methanol, 5·0 ml of 0·01 N-aqueous iodine solution (KI_3) was added. The reaction mixture was gently shaken

in a stoppered vessel for 10 min at room temperature (20 to 25°) and then titrated with standard 0·005 N-thiosulphate. Starch may be used as an indicator, but titration to the last trace of iodine colour is frequently more satisfactory (SIGGIA and EDSBERG, 1948).

(h) *Other methods*. Our modification of the *p*-nitrophenylhydrazone procedure of WITTENBERG, KOREY, and SWENSON (1956) for higher fatty aldehyde has been described (RAPPORT and ALONZO, 1955*b*). Other analytical methods in use in this laboratory are listed in earlier publications (RAPPORT and ALONZO, 1955*a*; RAPPORT, *et al.*, 1957).

RESULTS

Table 1 shows the compounds and fractions which were studied for addition of iodine under the conditions of SIGGIA and EDSBERG, for addition of bromine under the conditions of TRAPPE, and for liberation of higher fatty aldehyde by the method of WITTENBERG *et al.* Some of the fractions were also examined for addition of hydrogen.

The first point to be established is that methanolic iodine under the conditions described by SIGGIA and EDSBERG does not add to unsaturated bonds of the usual olefinic variety. This is clearly seen from preparations 1, 4, 5, and 13 in which the presence of such bonds in compounds analogous to those under study is established by addition of both hydrogen and bromine and does not result in uptake of iodine. The second point is confirmation of specificity with respect to acetal derivatives and α,β-unsaturated ethers. The results obtained with preparations 2 and 3 show that neither glyceryl acetals of higher fatty aldehydes nor the more rapidly hydrolysable dimethyl acetals react with iodine. Determinations carried out with preparations 15, 16, and 17 show that addition of iodine occurs not only with vinyl ethers but also with somewhat less reactive α,β-unsaturated ethers (dihydropyran). In our hands, the addition of iodine, although precise, is less accurate than described by SIGGIA and EDSBERG, but it is not certain whether this is to be attributed to impurity of the compounds or, more probably, to interference resulting from concomitant reactions which these very reactive compounds undergo. In any case, the reaction is sufficiently accurate to confirm the structural analogy between α,β-unsaturated ethers and plasmalogens.

Turning now to plasmalogens, preparation 6, lysophosphatidal ethanolamine from bovine *muscle*, is the crystalline compound ('acetal phospholipid') whose study provided conclusive evidence for the presence of the aldehydogenic linkage as an α,β-unsaturated ether. For this compound, iodine addition agrees well with the determination of fatty aldehyde content by formation of the *p*-nitrophenylhydrazone. The deviation from the theoretical value of 1 taken in conjunction with the very satisfactory analysis obtained for this compound (RAPPORT *et al.*, 1957) again suggests that competing reactions are responsible for the failure of these methods to be accurate. In any case, the degree of ambiguity is limited to the order of 10 per cent. A similar preparation of lysophosphatidal ethanolamine from *brain* (no. 7), yields essentially the same result; it contains a single double bond and this is of the α,β-unsaturated ether type. By concentrating the ethanolic mother liquor from this brain fraction and adding an equal volume of ethyl ether, preparation 8 was obtained. This fraction, colourless as well as crystalline, contains some olefinic unsaturation in addition to α,β-unsaturated ether, since bromination and hydrogenation show an excess of one third of a double bond over that required by the aldehydogenic linkage. The lysoethanolamine plasmalogens therefore contain olefinic aldehydogenic chains, but the

TABLE 1.—ANALYSIS OF UNSATURATION AND ALDEHYDE CONTENT OF PLASMALOGENS AND RELATED COMPOUNDS

Prep. No.	Description	Analysis	Fatty Aldehyde	Iodination	Bromination	Hydrogenation
			moles per mole*	moles I_2 per mole*	moles Br_2 per mole*	moles H_2 per mole*
1	Oleic acid		0	0	1·02	
2	Palmityl dimethyl acetal		0·85†	0	0	
3	Myristyl glyceryl acetal		0·48†	0	0	
4	Egg lecithin	‡	0	0	1·70	1·82
5	Vegetable lecithin	§	0·01	0	2·76	
6	Lysophosphatidal ethanolamine (bovine muscle)	P, 6·53	0·86	0·84	0·92	0·98
7	Lysophosphatidal ethanolamine (bovine brain)	P, 6·39	0·92	0·88	0·92	
8	Lysophosphatidal ethanolamine (bovine brain)	P, 6·49	0·91	0·86	1·29	1·33
9	Lysophosphatidal choline (bovine heart)	P, 5·95	0·88	0·93	1·00	
10	Beef heart lecithin (1 preparation)	P, 4·02	0·47	0·51	2·53	2·52
11	Beef heart lecithin (average of 7 preparations)	‖	0·52	0·54	2·49¶	
12	Lysophosphoglycerides prepared by enzymic hydrolysis of beef heart lecithin	P, 5·83		0·47	0·69	0·65
13	Fatty acids isolated after enzymic hydrolysis of beef heart lecithin	neutral equivalent = 302		0	1·77	2·06
14	Beef muscle kephalin	P, 3·87	0·62	0·67	3·64	3·75
15	Vinyl 2-ethyl hexyl ether			0·97	0·98	
16	Vinyl *iso*butyl ether			0·95		
17	Dihydropyran			0·93	0·96	

* Based on weight for preparations 1, 2, 3, 15, 16, 17; on phosphorus for preparations 4, 5, 6, 7, 8, 9, 10, 11, 12, 14; on neutral equivalent for preparation 13.

† 20 min at 70°, 0·1 N-acid. Optimal conditions not determined. WITTENBERG *et al.* (1956) report 0·51 for myristyl glyceryl acetal under these conditions, 0·88 with 0·4 N-acid for 90 min.

‡ RAPPORT and ALONZO (1955*a*). Table 1, preparation 1.

§ RAPPORT and ALONZO (1955*a*). Table 1, preparation 3.

‖ RAPPORT and ALONZO (1955*b*). Table 1, preparations 1, 3, 5, 6, 7, 8, and 9.

¶ Method of YASUDA.

fractions containing them are more soluble and may therefore be lost during purification.

The presence of the α,β-unsaturated ether linkage in *native* plasmalogens is shown by comparing the values for iodine addition and fatty aldehyde content (Table 1, columns 4 and 3) for preparations nos. 10, 11, and 14. Preparation no. 14 is an

ethanolamine phospholipid fraction from bovine skeletal muscle, no. 10 is a preparation of beef heart lecithin, whereas the results presented as no. 11 are the averages for 7 beef heart lecithins, complete analyses of which are to be found in an earlier publication (RAPPORT and ALONZO, 1955*b*). Enzymic hydolysis of beef heart lecithin with snake venom lecithinase A produces unsaturated free fatty acids (preparation no. 13) and a mixture of lysophosphoglycerides, preparation no. 12; only the latter contains the α,β-unsaturated ether grouping. This confirms results previously deduced from colorimetric determinations with fuchsin-sulphurous acid reagent indicating that phosphatidal choline in PANGBORN's beef heart lecithin has the unsaturated ether chain linked almost exclusively to the secondary hydroxyl group of the glycerol residue (RAPPORT and FRANZL, 1957; FRANZL and RAPPORT, 1955). A slight (10 per cent) loss in plasmalogen content of these lysophosphoglycerides obtained from beef heart lecithin may be ascribed to losses in purification with consequent enrichment of the mixture in its lysophosphatidyl choline content. Crystalline lysophosphatidal choline (preparation no. 9) obtained by alkaline hydrolysis of beef heart lecithin also conforms to the other plasmalogens in correspondence between iodine uptake and fatty aldehyde liberation. Ordinary olefinic unsaturation is almost absent from this preparation.

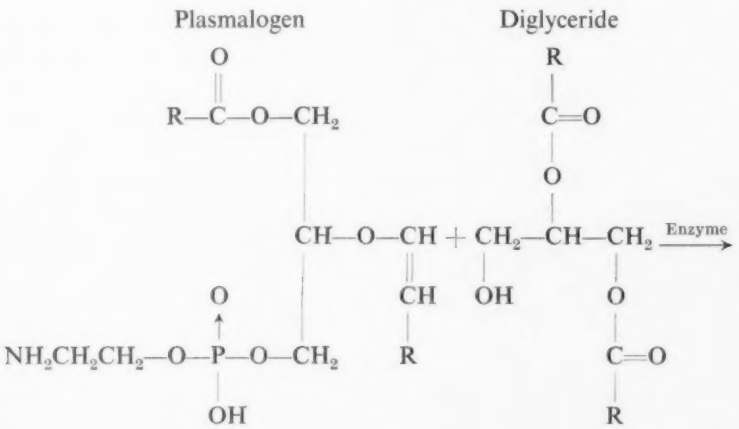
DISCUSSION

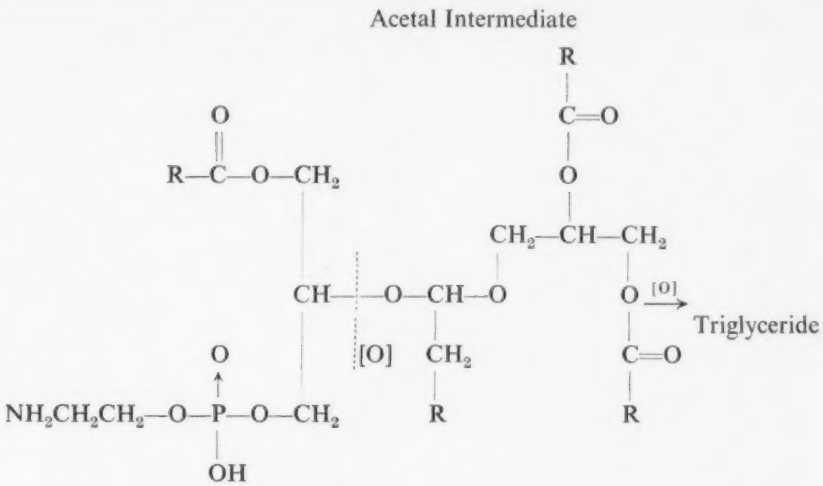
Three points are established by the observations reported in this paper. First, iodination in methanol is able to differentiate the unsaturation in α,β-unsaturated ethers from normal olefinic unsaturation. Second, plasmalogens subjected to several chemical and physical reactions (solvent extraction, enzymic hydrolysis, chromatographic adsorption before and after alkaline hydrolysis) add iodine and yield higher fatty aldehyde in molecular equivalents. This reaction, therefore, cannot be considered to result from artifacts arising from isolation procedures. And finally, plasmalogens from several tissues (skeletal muscle, heart, and brain) and containing either ethanolamine or choline as the nitrogenous base react with equivalent quantities of iodine and *p*-nitrophenylhydrazine. It seems, therefore, reasonably certain that the α,β-unsaturated ether formulation of the aldehydogenic linkage is correct for all naturally occurring plasmalogens which give rise to higher fatty aldehydes on hydrolysis, and that this structure is not limited to material either isolated by some unique method of fractionation or derived from some special tissue.

An α,β-unsaturated ether has not heretofore been detected in natural products from any source; it is therefore remarkable to find this very reactive radical widely distributed in a class of compounds, phosphoglycerides, whose relative lack of reactivity has resulted in their metabolic function remaining an enigma. From the standpoint of chemical reactivity the new structure is exceptionally provocative, since the reactions in which it can reasonably be expected to participate are innumerable. This degree of reactivity is reflected in the rapidity with which plasmalogens are hydrolyzed with weak acids. For example, at pH 2 (20 per cent acetic acid) the unsaturated ether linkage in lysophosphatidal ethanolamine is hydrolysed to the extent of more than 90 per cent in 30 min at 37°C (RAPPORT and LERNER, unpublished). The chemistry of α,β-unsaturated ethers in which the unsaturation is part of a long aliphatic chain is not yet known. For information we must turn to the chemistry of vinyl ethers and α,β-unsaturation in certain special ethers such as dihydropyran. The reactions of vinyl ethers are a subject of current study by organic chemists. A

survey (COPENHAVER and BIGELOW, 1949) shows that these include addition reactions, hydrolysis, and polymerization with acidic catalysts. Addition reactions are most striking since the unsaturated bond in vinyl (or α,β-unsaturated) ethers has increased reactivity compared with normal olefinic unsaturation, in contrast to that in vinyl (or α,β-unsaturated) esters or acids, where the presence of the electron-attracting carbonyl group produces a decrease in reactivity. Among the reactions listed in the survey (*loc. cit.*) are addition of hydrogen, alcohols, acetals, hydrogen halides, carboxylic acids, halogens, hydrogen sulphide, ammonia, amines and oxygen. Addition of water produces aldehyde *via* the hemi-acetal intermediate, and the probability is high that the hemi-acetal intermediate may actively engage in condensation reactions under special circumstances. Vinyl ether-alcohol interchange, catalysed by mercuric sulphate, has been reported to occur with reasonable speed at temperatures as low as $-78°$C (ADELMAN, 1955), attesting to the exceptional nature of the reactivity in these compounds. It seems a safe conjecture from considerations of constitution, distribution, and reactivity, that plasmalogens will prove to have a number of biochemical functions, depending on which enzyme systems are present in a particular tissue or site of action. In this respect, the degree of reactivity represented by these structures is akin to the 'high energy bond' of adenosinetriphosphate, and it is interesting that the tissues which are richest is plasmalogens are those with particularly high energy requirements, such as brain and muscle.

Although the addition reactions listed above indicate that any compound containing a free alcohol, amine, or sulphydryl group is a potential reactant with plasmalogens, chemical studies in the current literature (PARHAM and ANDERSON, 1948; TCHOUBAR, 1953; ADELMAN, 1955) tend to focus attention on those containing a free hydroxyl group. Such compounds readily combine with α,β-unsaturated ethers to form mixed acetals, which are themselves reactive intermediates. They may be hydrolysed to regenerate the free hydroxyl group and free aldehyde under relatively mild conditions. Potentially they may be oxidized to form esters, and in this connection, biochemical evidence implicating plasmalogens in processes associated with deposition of neutral fat (MÖCKEL, 1943; YARBRO and ANDERSON, 1956) is very suggestive. A possible mechanism for proceeding from a di- to tri-glyceride (or glycerol to mono-glyceride or mono- to di-glyceride) is the following:





Oxidation of the acetal produces an ester.

A second example consistent with this mechanism of esterification via acetal formation is suggested by studies of *in vitro* esterification of serum cholesterol (LE BRETON and PANTALÉON, 1947) in which phospholipids were reported to be hydrolysed concomitantly. Plasmalogens in serum are intimately associated with cholesterol and lecithin and may act as intermediates.

It must be emphasized that these examples are cited because of their capacity to illustrate a potential function of plasmalogens rather than to indicate that knowledge of structure is evidence for function at this early stage. Our ability to study plasmalogens by histochemical methods should be of considerable assistance in clarifying their biochemical roles, and in this respect, the study of this class of substances offers a valuable opportunity for relating chemical, biochemical, and biological information.

As a final thought, it is perhaps worth-while to point out that the presence of an α,β-unsaturated ether in a *phosphoglyceride* is important not only from the standpoint of its exceptional reactivity, but also from considerations of the relationship of spatial orientation to cellular integration. Our evidence suggests that the ether chain is attached to the β-hydroxyl group of the glycerol residue. This would permit alternate polar (phosphorylethanolamine) and non-polar (fatty ester) chains, aligned in the same direction, to interact with protein and structural lipids to produce oriented patterns of reactive unsaturated ether groups. The possible role of plasmalogens in active transport across membranes is not an unreasonable corollary.

SUMMARY

Analysis of the unsaturation in plasmalogens by iodination, bromination, and hydrogenation shows that although these substances may contain several double bonds, the quantity of iodine taken up under conditions specific for α,β-unsaturated ethers is equivalent to the quantity of fatty aldehyde liberated on acid hydrolysis. The α,β-unsaturated ether linkage in this class of compounds is thus confirmed. All plasmalogens examined behaved uniformly in this respect, whether derived from

muscle or brain, or containing ethanolamine or choline as the nitrogenous residue. Lysoplasmalogens formed by either alkaline or enzymic hydrolysis retain their α,β-unsaturated ether reactivity.

The chemical reactions and potential biochemical role of this structure, unique among natural products, is discussed.

Acknowledgements—We wish to thank Dr. C. L. YARBRO for the specimen of palmityl dimethyl acetal, Dr. J. B. WITTENBERG for the myristyl glyceryl acetal, Mr. S. ROSENBERG of the Sylvana Chemical Co. for egg lecithin and beef heart lecithin preparations.

This study was supported in part by a research grant (no. C-2316) from the National Cancer Institute of the National Institutes of Health, Public Health Service.

REFERENCES

ADELMAN R. L. (1955) *J. Amer. chem. Soc.* **77,** 1669.
COPENHAVER J. W. and BIGELOW M. H. (1949) *Acetylene and Carbon Monoxide Chemistry* pp. 40–46. Reinhold, New York.
FEULGEN R. and BERSIN T. (1939) *Hoppe-Seyl. Z.* **260,** 217.
FEULGEN R. and VOIT K. (1924) *Pflüg. Archiv. ges. Physiol.* **206,** 389.
FRANZL R. E. and RAPPORT M. M. (1955) *Fed. Proc.* **14,** 213.
HANAHAN D. J., TURNER L. D. and JAYKO M. E. (1951) *J. biol. Chem.* **192,** 623.
LE BRETON E. and PANTALÉON J. (1947) *Arch. sci. physiol.* **1,** 199.
MEAD J. F. and HOWTON D. R. (1950) *Anal. Chem.* **22,** 1204.
MÖCKEL G. (1943) *Hoppe-Seyl. Z.* **277,** 135.
PANGBORN M. C. (1951) *J. biol. Chem.* **188,** 471.
PARHAM W. E. and ANDERSON E. (1948) *J. Amer. chem. Soc.* **70,** 4187.
RAPPORT M. M. and ALONZO N. (1955*a*) *J. biol. Chem.* **217,** 193.
RAPPORT M. M. and ALONZO N. (1955*b*) *J. biol. Chem.* **217,** 199.
RAPPORT M. M. and ALONZO N. (1956) *Biochemical Problems of Lipids* (Ed. G. POPJÁK and E. LE BRETON) Butterworths, London. p. 69.
RAPPORT M. M. and FRANZL R. E. (1957) *J. biol. Chem.* **225,** 851.
RAPPORT M. M., LERNER B. and ALONZO N. (1954) *Fed. Proc.* **13,** 278.
RAPPORT M. M., LERNER B., ALONZO N. and FRANZL R. E. (1957) *J. biol. Chem.* **225,** 859.
SIGGIA S. and EDSBERG R. L. (1948) *Anal. Chem.* **20,** 762.
TCHOUBAR B. (1953) *C. R. Acad. Sci., Paris* **237,** 1006.
THANNHAUSER S. J., BONCODDO N. F. and SCHMIDT G. (1951) *J. biol. Chem.* **188,** 417.
TRAPPE W. (1938) *Biochem. Z.* **296,** 180.
WITTENBERG J. B., KOREY S. R. and SWENSON F. H. (1956) *J. biol. Chem.* **219,** 39.
YARBRO C. L. and ANDERSON C. E. (1956) *Proc. Soc. exp. Biol. Med.* **91,** 408.
YASUDA M. (1931) *J. biol. Chem.* **94,** 401.

VOL
1
1956/

Journal of Neurochemistry, 1957, Vol. 1, pp. 311-315, Pergamon Press Ltd., London

HISTOCHEMICAL STUDIES ON MONOAMINE OXIDASE IN THE MID-BRAIN OF THE MOUSE

IWAO ARIOKA and HIROSHI TANIMUKAI
Department of Neuropsychiatry, Osaka University Medical School, Osaka-Fukushima, Japan

(*Received* 12 *February* 1957; in revised form 21 *March* 1957)

ALTHOUGH monoamine oxidase is widely distributed in animal and human organs (BHAGVAT, BLASCHKO and RICHTER, 1939; BLASCHKO, RICHTER and SCHLOSSMANN, 1937; BOGDANKSI and UDENFRIEND, 1955, 1956; HOLTZ, HEISE and SPREYER, 1938; PUGH and QUASTEL, 1937*a*) and is believed to play an important role in the detoxication of toxic amines (PEARSE, 1953; ZELLER, 1951), few histochemical studies on the distribution of this enzyme have been reported (FRANCIS, 1953; KOELLE and VALK, 1954; OSTER and SCHLOSSMAN, 1942), and there are no data on its histological localization in the brain, since no reliable histochemical method for demonstrating this enzyme in the central nervous system has been obtained. In recent studies (BLASCHKO and HELLMANN, 1953; BLASCHKO and PHILPOT, 1953; SJOERDSMA, SMITH, STEVENSON and UDENFRIEND, 1956; UDENFRIEND and TITUS, 1954; UDENFRIEND, TITUS, WEISSBACH and PETERSON, 1956) it has come to light that serotonin (5-hydroxytryptamine) is oxidized by monoamine oxidase with the formation of dark brown pigments. By utilizing this reaction, the present authors have now developed a histochemical technique for demonstrating monoamine oxidase activity in the tissues, and we have studied the distribution of the enzyme in the midbrain of the mouse.

MATERIALS AND METHODS

All animals used in this investigation were male adult NA-I mice kept at room temperature. It was found that young animals and mice of other genetic strains showed lower amine oxidase activities. The animals were killed by decapitation, and as soon as the brain was removed, a slice of the mid-brain (0·5 mm in thickness) was rapidly taken and the following procedures were carried out:

(1) The fresh unfixed slice of the brain was washed in M/15 phosphate buffer (pH 7·4) for a moment.

(2) It was incubated for 3 to 8 hr at 37°C, oxygenating every 15 min, in an incubation medium containing: 5 mg/10 ml 5-hydroxytryptamine creatinine sulphate solution (pH 7·4). It was found that traces of heavy metals (Mo, Cu, Fe, Co, etc.) in the serotonin preparation had an accelerating effect on the pigment formation.

(3) The slice was washed in water.

(4) It was embedded in paraffin.

(5) Sections were cut, deparaffinized and the nuclei were stained with Mayer's haemalum if desired.

(6) The preparation was dehydrated in alcohol, cleared in xylene and mounted in cedar oil.

Sites of monoamine oxidase activity in the tissue are shown by the deposition of a dark brown pigment.

(7) The two following controls were performed:

(a) Control tissue incubated in the buffer solution without the substrate.

(b) Control tissue subjected to the above procedures after the enzyme has been inactivated by heating at 80°C for 30 min.

No pigment formation was observed in the controls.

RESULTS

In general, the dark brown coloration indicating monoamine oxidase activity was heavier in the grey matter and less in the white matter of the brain. The coloration of the hypothalamus was so intense that it appeared dark brown without magnification.

Microscopically, the pigment granules were seen in the cytoplasm, especially in the perinuclear region, and absent in the nuclei, of nerve cells of the paraventricular structures (Figs. 1 and 2).

The cytoplasm of the ependymal cells and the epithelial cells and stroma of the choroid plexus showed an intense positive reaction (Figs. 3 and 4), as also did the capillary walls (Fig. 5).

No pigment formation was found in the nerve fibres or in the glial cells.

The problem of which basal nuclei in the hypothalamus contain the enzyme has been investigated and the results will be presented separately.

DISCUSSION

1. *Evaluation of the specificity of the histochemical method*

An attempt to demonstrate the monoamine oxidase in tissues histochemically was first made by OSTER and SCHLOSSMAN (1942). Their method, however, has been criticized by GOMORI (1950, 1953) and by PEARSE (1953) in view of its incomplete specificity and unsatisfactory localization. FRANCIS's method (1953) cannot be recommended, because the coloration also developed on incubating the tissue in the solution without the substrate. KOELLE and VALKS's method (1954) was reliable, but not available for practical studies owing to the difficulty of obtaining the reagents and the complicated nature of the procedures. In short, it appeared that a practical method for the histochemical demonstration of monoamine oxidase had not been obtained.

In the biochemical field PUGH and QUASTEL (1937*a*,*b*) observed that melanin-like pigments firmly bound to the tissue were formed by the oxidation of indolethylamine when it was incubated with tissues, and they suggested that this reaction might be catalysed by monoamine oxidase. BLASCHKO and HELLMANN (1953) confirmed that tryptamine was oxidized by monoamine oxidase, and that it passed through the aldehyde and finally formed dark brown pigments insoluble in water; they reported that this reaction was available as a histochemical technique and that it occurred similarly when 5-hydroxytryptamine was used.

Recently it became known (BLASCHKO and PHILPOT, 1953; SJOERDSMA *et al.*, 1956; UDENFRIEND and TITUS, 1954; UDENFREIND *et al.*, 1956) that serotonin is deaminated by the monoamine oxidase in the tissues to give 5-hydroxyindoleacetaldehyde, which is excreted in the urine as 5-hydroxyindoleacetic acid. It was also made clear that serotonin has another metabolic pathway to form the dark brown pigment from 5-hydroxyindoleacetaldehyde (Fig. 6).

It should therefore be possible to show the activity of the monoamine oxidase in the tissues by observing the pigment formation when serotonin is used as substrate. This pigment formation, which occurs under aerobic conditions, is inhibited by various substances such as urea, pentamidine, octylalcohol, and 1-*iso*nicotinyl-2-*iso*propylhydrazide: it is also inhibited by semicarbazide which as a carbonyl reagent prevents further oxidation of the aldehyde. In our own experiments, the pigment formation was not seen when the tissue was previously boiled or when serotonin alone was

VOL.
1
1956/5

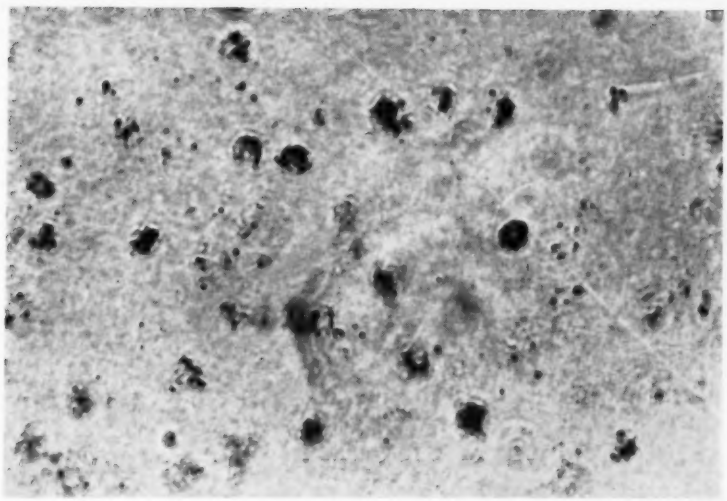


FIG. 1.—Pigment granules formed from serotonin in the hypothalamus. Monoamine oxidase activity, which is indicated by the dark brown pigments, is seen in the cytoplasm of nerve cells. A few dark particles are observed in the intercellular space: these are attributed to diffusion. No counterstain.

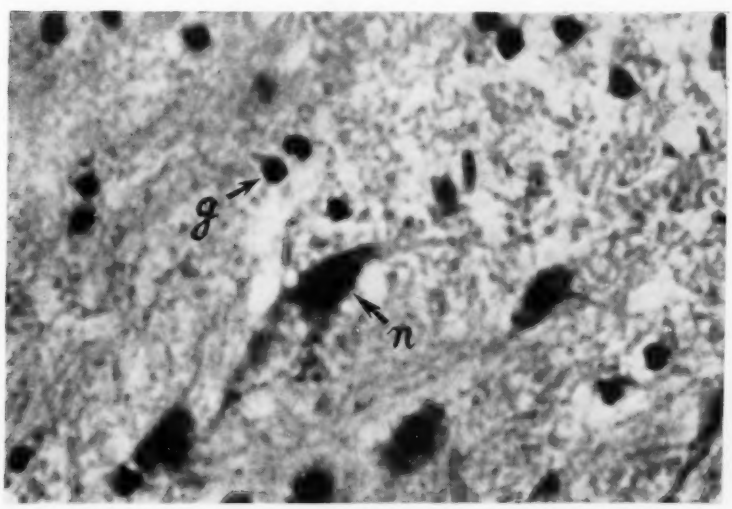


FIG. 2.—Nerve cells and glial cells in the hypothalamus. Monoamine oxidase activity is seen in the cytoplasm of the nerve cells, but is absent in the nuclei. No monoamine oxidase activity is observed in glial cells. The inserted arrows indicate examples of a nerve cell (*n*) and a glial cell (*g*).
Nuclei counterstained with Mayer's haemalum.

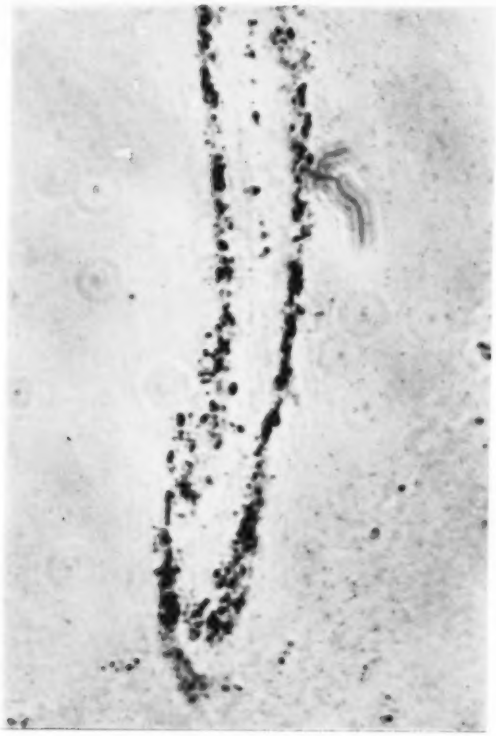


FIG. 3.—Ependyma of the third ventricle. Monoamine oxidase activity is seen in the cytoplasm of ependymal cells. No counterstain.

FIG. 4.—Plexus choroideus. Monoamine oxidase activity is found in the cytoplasm of the epithelial cells and in the stroma. Arrows indicate the pigment formed from serotonin. Nuclei counterstained with Mayer's haemalum.

VOL
1
1956/

OL.
1
6/57

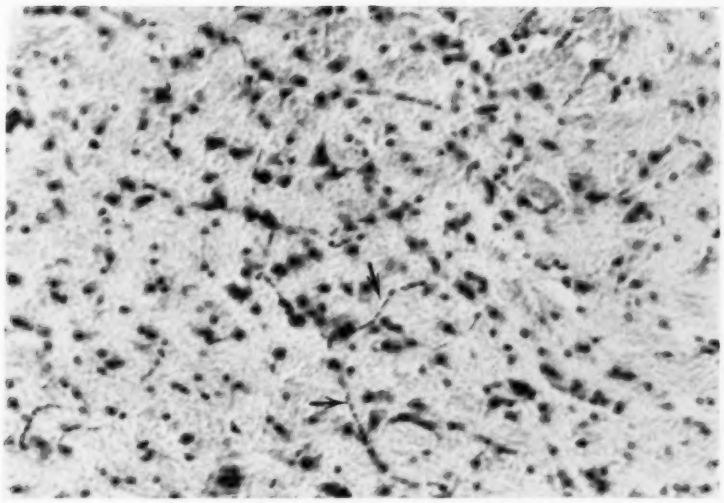
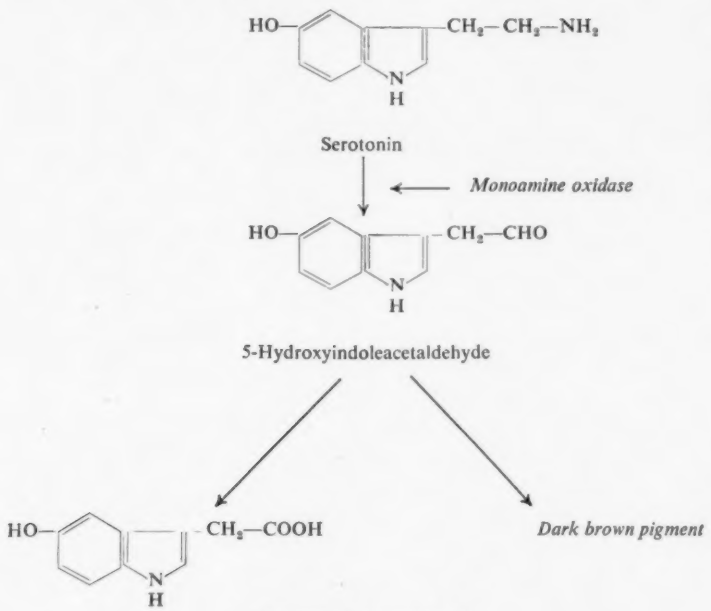


FIG. 5.—Hypothalamus. Arrows indicate the capillary walls which contain the monoamine oxidase. Counterstained with Mayer's haemalum.

VOL.
1
1956/5

treated by hydrogen peroxide *in vitro*. From these facts, it is concluded that the formation of dark brown pigments from serotonin under aerobic conditions is catalysed by the monoamine oxidase. The intensity of the pigment formation, however, does not always indicate the quantity of the enzyme.

According to BLASCHKO and HELLMANN (1953), the pigment is rapidly dissolved by organic solvents, such as ethanol, acetone, dioxane and *n*-butylalcohol, so that it is difficult to use this reaction for histochemical purposes. But in our experiments it appeared that the pigment formed from serotonin was relatively insoluble in ethanol, acetone, *n*-butylalcohol and formalin.



5-Hydroxyindoleacetic acid.

FIG. 6

2. *The distribution of the monoamine oxidase in the brain*

There have been no previous histochemical studies of the monoamine oxidase in the brain. In our studies, the enzymic activity was found to be higher (a) in the hypothalamus (b) in the cytoplasm of nerve cells, ventricular ependymal cells and capillary wall cells, and (c) in the epithelial cells and stroma of the choroid plexus. Recently, BOGDANSKI and UDENFRIEND (1955, 1956) reported that the monoamine oxidase activity was highest in the hypothalamus of the dog brain, as indicated by biochemical estimation. The present histochemical results agree therefore with their findings.

It is believed that monoamine oxidase is concerned in the detoxication of toxic amines produced in the body and the destruction of serotonin and adrenaline (BHAGVAT *et al.*, 1939; HARTUNG, 1946; RICHTER and TINGEY, 1939).

Intravenously injected serotonin does not affect the brain of rabbits and dogs (UDENFRIEND, WEISSBACH and BOGDANSKI 1957) or mice (SHORE *et al.*, 1957), and no

increase in serotonin concentration was found in the cerebrospinal fluid of patients with malignant carcinoid whose blood contained twenty times the normal concentration of serotonin (SJOERDSMA, WEISSBACH and UDENFRIEND, 1956). It is therefore believed that serotonin cannot permeate the blood-brain barrier. According to many authors (FRIEDEMANN, 1937, 1942; MAYER and BAIN, 1956; NIESSING, 1952; SPATZ, 1934; WALTER, 1934), the blood-brain barrier depends on the intracerebral capillary walls, and the blood-cerebrospinal fluid barrier depends on the epithelial cells and stroma of the choroid plexus. In view of these facts and the present observations, the authors draw attention to the possibility that the monoamine oxidase may play a role in the so-called blood-brain barrier function.

Biochemical estimations have shown that serotonin is abundant in the hypothalamus (AMIN, CRAWFORD and GADDUM, 1953; BOGDANSKI and UDENFRIEND, 1955, 1956) and the present histochemical experiments have demonstrated that the monoamine oxidase, which deaminates serotonin, is also abundant in the hypothalamus. These observations suggest that the monoamine oxidase may regulate the actions of serotonin in the nerve cells.

SUMMARY

(1) A new histochemical method for showing monoamine oxidase activity in the tissues has been described. This depends on the formation of dark brown pigments from 5-hydroxytryptamine (serotonin) under aerobic conditions. The specificity and definitions of the method are discussed.

(2) A histochemical study of the distribution of monoamine oxidase activity in the mid-brain of the mouse was made by this method. It was found that activity was present (a) in the grey matter, especially in the hypothalamus, (b) in the cytoplasm of nerve cells, ventricular ependymal cells and capillary wall cells, and (c) in the epithelial cells and stroma of the choroid plexus.

(3) The authors draw attention to the possibility that the monoamine oxidase may play a role in relation to the blood-brain barrier function and may also regulate the actions of serotonin in the nerve cells.

REFERENCES

AMIN A. H. T., CRAWFORD B. B. and GADDUM J. H. (1953) *Abst. XIX Internat. Physiol. Congress Montreal* p. 165.

BHAGVAT K., BLASCHKO H. and RICHTER D. (1939) *Biochem. J.* **33,** 1338.

BLASCHKO H. (1952) *Biochem. J.* **52,** 10.

BLASCHKO H., RICHTER D. and SCHLOSSMANN H. (1937) *Biochem. J.* **31,** 2187.

BLASCHKO H. and HELLMANN K. (1953) *J. Physiol.* **122,** 419.

BLASCHKO H. and PHILPOT F. J. (1953) *J. Physiol.* **122,** 403.

BOGDANSKI D. F. and UDENFRIEND S. (1955) *Abst. Amer. Soc. Pharm. exp. Therap. Inc.* Iowa, Sept. 6–8.

BOGDANSKI D. F. and UDENFRIEND S. (1956) *J. Pharmacol.* **116,** 7.

ERSPAMER V. (1954) *Experientia* **10,** 471.

FRANCIS C. M. (1953) *Nature, Lond.* **171,** 701.

FRIEDEMANN U. (1937) *J. Immunol.* **32,** 97.

FREIDEMANN U. (1942) *Physiol. Rev.* **22,** 125.

GOMORI G. (1950) *Acad. Sci.* **50,** 968.

GOMORI G. (1953) *Microscopic Histochemistry* Chicago.

HARTUNG W. H. (1946) *Ann. Rev. Biochem.* **15,** 593.

HOLTZ P., HEISE R. and SPREYER W. (1938) *Arch. exp. Path. Pharmak.* **188,** 580.

KOELLE G. B. and VALK A. T. (1954) *J. Physiol.* **126,** 434.

VOL.
1
1956/5

MAYER S. E. and BAIN J. A. (1956) *J. Pharmacol.* **118,** 17.
NIESSING K. (1952) *Dtsch. Z. Nervenheilk.* **168,** 485.
OSTER K. A. and SCHLOSSMAN N. C. (1942) *J. cell. comp. Physiol.* **20,** 373.
PEARSE A. G. E. (1953) *Histochemistry* London.
PUGH C. E. M. and QUASTEL J. H. (1937*a*) *Biochem. J.* **31,** 286.
PUGH C. E. M. and QUASTEL J. H. (1937*b*) *Biochem. J.* **31,** 2306.
RICHTER D. and TINGEY A. H. (1939) *J. Physiol.* **97,** 265.
SHORE P. A., PLETSCHER A., TOMICH E. G., CARLSSON A., KUNTZMAN R. and BRODIE B. B. (1957) *Ann. N.Y. Acad. Sci.* (In press).
SJOERDSMA A., SMITH T. E., STEVENSON T. P. and UDENFRIEND S. (1956) *Proc. Soc. exp. Biol. Med.* **89,** 36.
SJOERDSMA A., WEISSBACH H. and UDENFRIEND S. (1956) *Amer. J. Med.* **22,** 520.
SPATZ H. (1934) *Arch. Psychiat.* **101,** 267.
UDENFRIEND S. and TITUS E. D. (1954) *Fed. Proc.* **13,** 411.
UDENFRIEND S., TITUS E. D., WEISSBACH H. and PETERSON R. E. (1956) *J. Biochem.* **219,** 335.
UDENFRIEND S., WEISSBACH H. and BOGDANSKI D. F. (1957) Personal communication.
WALTER F. K. (1934) *Arch. Psychiat.* **101,** 195.
ZELLER E. A. (1951) *The Enzymes* Vol. II. Part I. p. 536. Academic Press, New York.

OL.
1
6/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 316-325, Pergamon Press Ltd., London

THE CHEMICAL CHANGES IN REGENERATING NEURONS

S.-O. BRATTGÅRD, J.-E. EDSTRÖM and H. HYDÉN
Department of Histology, The University of Gothenburg, Sweden

(*Received* 4 *March* 1957)

VOL
1
1956/

QUANTITATIVE ultramicro-methods now available permit the analysis of individual nerve cells. It is therefore possible to study more directly the primary processes concerned in the re-establishment of the neuron during regeneration.

Regeneration implies the outgrowth of a nerve cell process and its development into a functioning axon capable of conducting nerve impulses (WEISS, 1950, 1955). In this complex problem we have studied the correlation between the quantitative changes in the nerve cell components and the outgrowth of the axon, as well as the changes in the cell body volume (BRATTGÅRD, EDSTRÖM and HYDÉN, 1957). Since the method used in this study allows the cytology to be related to the cell chemistry, we have also studied the question of the cytochemical correlation of chromatolysis, known since the early days of cytology (NISSL, 1892; VAN GEHUCHTEN, 1897).

The restoration of the morphological continuity in the severed nerve involves a considerable production of cell protein (HYDÉN, 1943). In view of our present knowledge of protein formation special interest attaches to the relation between the ribonucleic acid (RNA) of the nerve cell and the production of protein (CASPERSSON, 1941; HYDÉN 1943, 1950). In this respect nerve regeneration can be considered also as a problem of growth and chemical differentiation.

MATERIAL AND METHODS

In all, 69 rabbits of 1·5 to 2·0 kg were used for the experiments. The hypoglossal nerve on one side was exposed and crushed with forceps cooled to -70°C at the point in the proximal third of the nerve, where it traverses the digastric muscle. Analyses were made from two to ninety days after crushing. The results from the regenerating cells were always compared with those obtained from the hypoglossal cells on the contralateral, unoperated side.

Morphologically, the hypoglossal nucleus is composed of different groups of cells (CAJAL, 1952; METTLER, 1948). The main part of the nucleus, however, consists of large nerve cells of practically uniform size and structure. We have used these cells exclusively and have investigated about three thousand of them. The hypoglossal nerve has only one maximum at 7–9 μ in its fibre spectrum (HÄGGQVIST, 1938). The part containing the hypoglossal nucleus was removed as rapidly as possible and frozen in liquid nitrogen.

Analysis by X-ray microradiography. The tissue was cut in a special cryostat at -20°C in 10 μ sections using a knife of a special steel, sharpened and controlled according to the method devised by HALLÉN (1956). The sections were mounted on 2 μ thick Al foil and dried at -40°C at a slight vacuum over P_2O_5 in a small freezer built into the cryostat. In this way dried sections from fresh nerve cells were obtained. This material was then exposed at 8–10 Å for the determination of the amount of dry organic substance divided into lipid and protein fractions, as has been described earlier (ENGSTRÖM and LINDSTRÖM, 1950; BRATTGÅRD and HYDÉN, 1952, 1954).

For the computation of the mass of whole, fresh and unfixed hypoglossal nerve cells of control rabbits the following procedure is used. The nerve cells are dissected out by hand in a Zeiss stereoscopic microscope a few minutes after killing the animal. In order to render the cells visible a drop

of very diluted methylene blue solution in 0·88 M-sucrose is applied on the section through the nucleus for approximately 2 min. The nerve cells then acquire a pale sky-blue colouring and can be dissected after 10 min. The free cells are sucked up in a micro-pipette and applied on a 1 μ thick Al foil of the preparation holder. From a drop of the solution containing the free cells each nerve cell is taken and placed on the Al foil covering the slit of the holder. Each cell is carefully and rapidly washed free from sucrose. It was possible during all these manipulations to avoid the micro-manipulator and thus shorten the time considerably until the cell material was exposed or frozen down.

The contact X-radiograms were evaluated photometrically (BOURGHARDT *et al.*, 1953) or by a recently constructed scanning cell analyser (BOURGHARDT, HYDÉN and NYQVIST, 1955; HYDÉN and LARSSON, 1957). This instrument measures the distribution and amount of dry mass from many cells in a short time.

The X-ray analysis gives results expressed as weight per unit area. In order to obtain the concentration value, the thickness of the section must be known. A new optical instrument for determining the thickness has recently been devised by HALLÉN (1956). Furthermore, in our present study all results are based on a comparison between the operated and the control material in the same section.

Determination of RNA. For the determination of the content and concentration of RNA of the individual hypoglossal cells the microchemical method worked out by EDSTRÖM (1953) was used. The principle of the method is to determine RNA in single Carnoy fixed cells photometrically on photographs taken after enzymic extraction.

The method for RNA determination was worked out for use on cells containing about 500 $\mu\mu$g (1 $\mu\mu$g = 10^{-12} g). Normal hypoglossal cells from the rabbit were found to contain about 200 $\mu\mu$g. To obtain an ideal range the control cells were generally analysed three at a time. At three periods (2, 15 and 48 days) single cells were taken from the control as well as from the operated side. Such a procedure demands a relatively large number of analyses. Three times four cells were investigated from each side at each period, and all cells taken from each animal were dissected from the same section, control and operated cells from symmetrical areas.

Determination of the cell volume. X-ray microradiography carried out with the precautions referred to above gives the organic mass concentration. To obtain the RNA concentration the volume of the cell body was determined. For the volume determinations two independent methods were applied on Carnoy fixed materials, and a third method was used to check the two first ones.

About two hundred determinations were carried out on Carnoy fixed material by optical reconstruction according to EDSTRÖM (1953) (see Table 2). The second method included one thousand eight hundred volume determinations on fixed cell sections. Since nerve cells, however, do not have an ideal shape for this method, a correction had to be made for the resulting error by using a three-dimensional reconstruction. The results from the two methods used showed satisfactory agreement.

The histological procedure, including the fixation, is reported to give volume shrinkage (STOWELL, 1941). ROBINS, SMITH and EYDT (1956) have studied especially the effect of formaldehyde fixation and found a volume shrinkage of about 80 per cent. For quantitative cytochemical studies BRATTGÅRD and HYDÉN (1952) emphasized the importance of using only untreated frozen material. Under certain circumstances, however, fixed cell material which has undergone volume changes is unavoidable. In such cases a correction for volume changes may be used, provided the volume change caused by the particular fixative has been estimated for the cell material studied. In the present study Carnoy fixation was used for the RNA determinations. The histological procedure, including fixation, was found to give an average shrinkage of 25 per cent in one dimension in a small piece of nervous tissue, i.e. 58 per cent volume decrease.

In order to obtain a correction factor for the volume change, the following method was used. Fresh hypoglossal cells were dissected in Ringer solution and the volume was determined as described above. When the volumes of the fresh cells were compared with those of the fixed cells it was found that the latter had undergone a shrinkage of 53 per cent, which agrees well with the 58 per cent given above. Data on nerve cells, in a condition as similar to the native state as can at present be attained, were obtained in this way.

Since, however, the main result obtained in our study is a change of the volume of the nerve cells on the operated side compared with that on the non-operated side (see Table 2, 3) such corrections do not affect the main conclusions.

Ultraviolet micrographs at 2570 Å were also made and sections were examined after staining with toluidine blue, methylene blue, gallocyanine and cresyl violet.

RESULTS

Control material. The results obtained with hypoglossal cells from control animals are given in Table 1. The mean volume of the hypoglossal cells was rather small. The individual fresh cell had a weight of 2770 $\mu\mu$g and a mass concentration of 0·20 $\mu\mu g/\mu^3$ which gives a volume of $13{\cdot}8 \times 10^3$ μ^3. The three values for the cell volume obtained by different methods, 13·3, 13·1, and $13{\cdot}8 \times 10^3$ μ^3 respectively, agree well. The main part of this consists of proteins and a smaller part (20 per cent or 550 $\mu\mu$g) of lipids extractable with chloroform. The amount of RNA averages 200 $\mu\mu$g. The concentration thus averages 1·5 per cent. This is a mean value which is in agreement with that found in other types of motor nerve cells fixed in Carnoy solution (EDSTRÖM, 1957). Fig. 1 shows four hypoglossal cells taken at 2570 Å. Fig. 2 shows a high-resolution radiogram taken at 8–10 Å of two Carnoy fixed hypoglossal cells. Cell details well below 1 μ in size are resolved.

TABLE 1.—ANALYSES OF HYPOGLOSSAL CELLS FROM CONTROL RABBITS

Volume of cell body	RNA	Dry weight	Lipids	Proteins	Axon mass
$13{\cdot}3 \times 10^3$ μ^3	200 $\mu\mu$g	2770 $\mu\mu$g	550 $\mu\mu$g	2200 $\mu\mu$g	0·06 $\mu\mu g/\mu^3$

Statistical analysis showed that there was no significant difference in volume, amount of protein, or RNA between the cells taken from the right and left hypoglossal nucleus. Furthermore, the cell values within each nucleus were distributed on a Gaussian curve. Thus, this material proved to be very suitable for our experiments.

Regeneration. The following division of the regenerating period into latent, outgrowth and maturation periods is based on anatomical and chemical data. In such a continuous phenomenon as nerve regeneration all limits with respect to time are naturally approximate.

(a) *The latent period* (1–2 days after nerve crush). During this first period, which is here called the latent period, degeneration of the central part of the nerve fibre occurs proximally to the place of the crush. An outgrowth of numerous thin processes can also be demonstrated a few hours after the trauma. This, however, is a transient phenomenon. Processes which can be defined structurally as axons appear on the second day in the degenerating central stump (CAJAL, 1928).

Our results (Tables 2 and 3; Figs, 3 and 4) show that during the first two days the volume of the cell body remains relatively constant, as does the amount and concentration of RNA. The dry cell substance, consisting mainly of proteins, decreases by 30 to 40 per cent.

Already one day after the nerve crush the absorption at 2600 Å characteristic of RNA is seen to decrease in a part of the cytoplasm. This change manifests itself during the first week and will eventually include all the cytoplasm with the exception of the parts near to the cell membrane (Figs. 5 and 6). Furthermore, it can be seen how the appearance of the absorbing aggregates containing RNA changed to a finely dispersed, faintly absorbing configuration.* If stained by the ordinary Nissl-staining

* The resolution of the u.v.-microscope using a monochromator 2·5 mm corrected for 2570 Å lies at 1000 Å.

VOL.
1
56/57

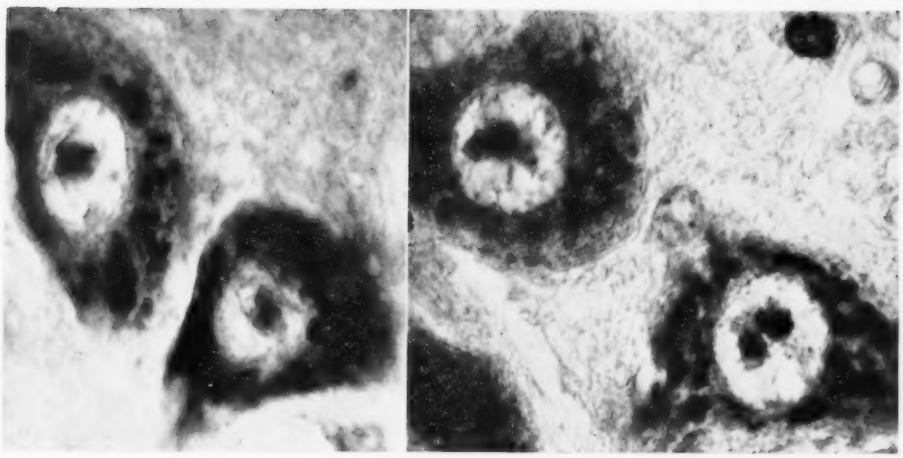


FIG. 1.—Hypoglossal cells from a control rabbit taken at 2570 Å. 2·5 mm monochromate, ocular 10×.

FIG. 2.—High-resolution radiogram taken at 8–10 Å on a Kodak spectrographic emulsion 649 Å of two Carnoy-fixed hypoglossal cells. Cell details well below 1 μ in size are resolved.

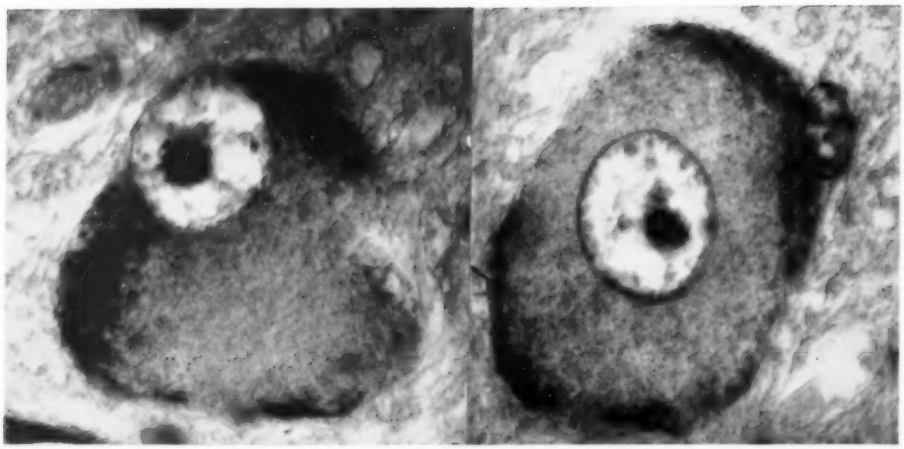
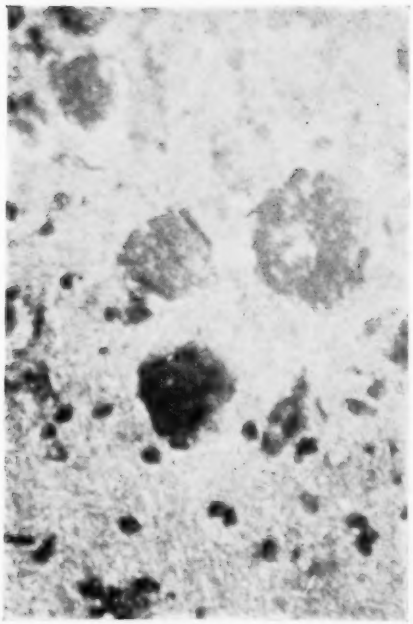


FIG. 5.—Two hypoglossal cells taken at 2570 Å one week after the operation. Ocular 10×.

VOL
1
1956/

VOL.
1
56/57



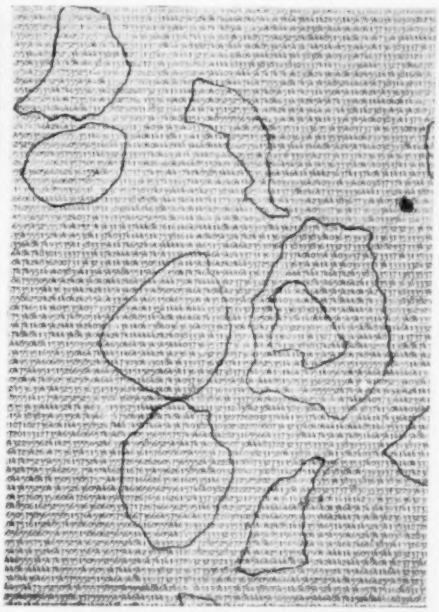


FIG. 6(a).—Hypoglossal cells stained with toluidine blue and photographed after the analyses in an ultro-pak system.

FIG. 6(b).—The same cells taken at 8–10 Å on a Kodak spectrographic emulsion 649 Å. Since the cell sections are taken from fresh, frozen and dried tissue no organic material has been dissolved from the nucleus of the cytoplasm. The nucleus therefore does not show up as a more or less empty space in the cell.

FIG. 6(c).—The picture in figures of the transmission values per 5 μ^2 of the same cells given by the cell analyser.

VOL
1
1956/

methods the well-known chromatolysis phenomenon can be seen. According to GERSH and BODIAN (1943) the changes in chromatolysis may be due to the depolymerization of RNA.

(b) *The outgrowth period* (3–12 days after the nerve crush). During this period thin new axons grow out through 'the degenerating and scar tissue' and the peripheral stump, and become connected with the motor end plates. On the fifth day after

TABLE 2.—ANALYSES OF RNA AND VOLUME OF CARNOY FIXED HYPOGLOSSAL CELLS

Days after op.	Cell body volume in 10^3 μ^3			$\mu\mu$g RNA per cell body		$\mu\mu$g RNA operated side/non-operated side	
	operated	non-operated	op/non-op.	operated	non-operated	per cell	per μ^3
2	6·1	6·1	1·0	159	171	0·93	0·93
4	10·2	5·0	2·0	180	198	0·91	0·46
5	10·1	4·7	2·1	—	—	—	—
6	12·2	4·7	2·6	184	199	0·92	0·35
8	12·5	6·0	2·1	—	—	—	—
9	11·8	5·3	2·2	240	236	1·02	0·50
15	11·7	5·6	2·1	242	164	1·48	0·70
27	14·9	6·1	2·4	380	183	2·08	0·87
48	13·2	5·4	2·4	469	216	2·17	0·90
77	9·1	6·9	1·3	295	228	1·29	0·99

TABLE 3.—ANALYSIS OF DRY WEIGHT, LIPIDS AND PROTEINS IN CELL BODIES OF FRESH HYPOGLOSSAL CELLS

Days after oper.	Volume operated / non-oper.	Mass/μ^3 in $\mu\mu$g		Lipids/μ^3 in $\mu\mu$g		Proteins/μ^3 in $\mu\mu$g		Proteins operated side/non-operated side*	
		operated	non-operated	operated	non-operated	operated	non-operated	per cell	per μ^3
0	1·00	—	0·20	—	0·04	—	0·16	1·00	1·00
2	1·10	0·14	0·21	0·05	0·05	0·09	0·16	0·62	0·56
3	1·40	—	—	—	—	—	—	—	—
4	1·69	0·15	0·21	0·05	0·05	0·10	0·16	1·06	0·63
5	2·30	—	—	—	—	0·09	0·17	1·22	0·53
6	2·60	—	—	—	—	0·10	0·17	1·53	0·59
7	2·47	—	—	—	—	0·12	0·17	1·73	0·70
8	2·30	0·15	0·21	0·03	0·05	0·12	0·16	1·73	0·75
15	2·20	0·15	0·20	0·03	0·04	0·13	0·16	1·78	0·81
30	2·50	0·20	0·21	0·04	0·05	0·16	0·16	2·50	1·00
42	2·50	—	—	—	—	—	—	(2·50)	(1·00)
90	1·10	—	—	—	—	—	—	(1·16)	(1·00)

* Proteins of operated side/non-operated side per cell and per μ^3.

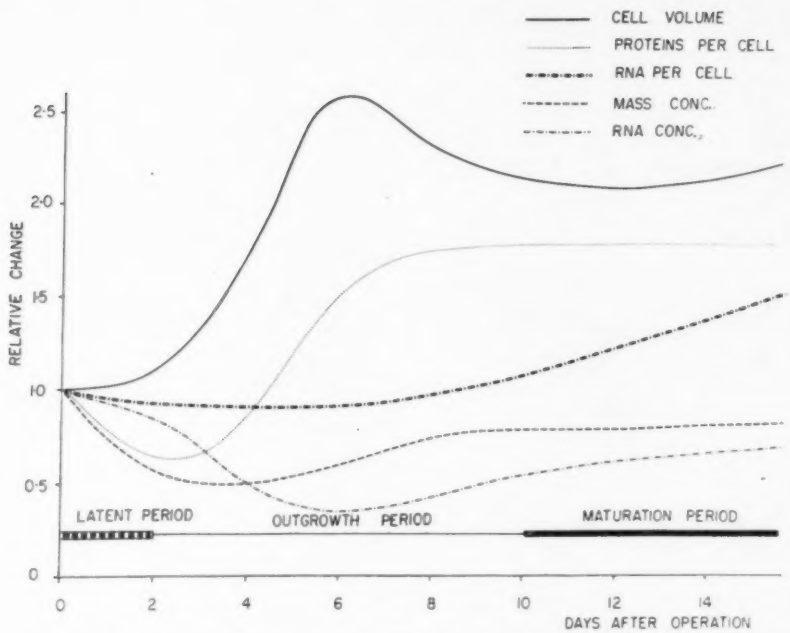


FIG. 3.—Showing the quantitative nerve cell changes occurring from 1 to 12 days after the operation.

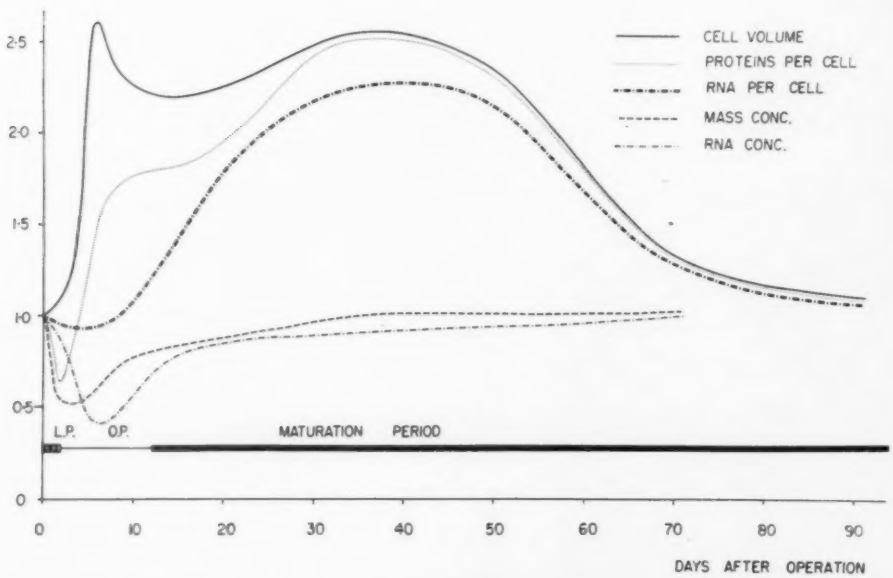


FIG. 4.—Showing the quantitative nerve cell changes occurring during the 1–90 days after the operation.

VOL
1
1956/

crushing, the axons can be detected in the peripheral part; the rate of outgrowth has been found to be around 4 mm per day for motor nerves, with very little variation (GUTMANN *et al.*, 1942). We have estimated the time for the completed outgrowth of the hypoglossal nerve in our experiments to be 12 to 13 days after the nerve crush.

During the third to sixth day the volume of the cell body increases to 2·6 times the original volume and, since the amount of RNA remains practically unchanged, the RNA concentration consequently decreases by about 60 per cent. The protein concentration remains low (Table 3). The net result is that the nerve cell body during these four days more than doubles its content of proteins and lipids.

During the rest of this period the cell body volume decreases moderately. The amount and concentration of RNA and of proteins and lipids, on the other hand increase to some extent, but not by more than 20 per cent (Tables 2, 3). The absorption at the nucleic acid maximum increases to a maximal value in most hypoglossal nerve cells.

(c) *The maturation period.* This period is characterized by the increase in the diameter of the newly established axons. The data presented covers in detail the first 2·5 months of this period. The axons grow slowly and, the diameter will return to its normal value within a year after the nerve crush (GUTMANN and SANDERS, 1943).

The full number of terminal branches in the motor endplates was reached about 90 days after the nerve crush (GUTMANN *et al.*, 1942). Signs of functional recovery were found within 12 days after the arrival of fibres at the endplates, i.e. 25 days after the nerve crush in our experiments.

Our results show a volume increase in the nerve cell body, reaching a maximum at 40 days, after which the volume slowly decreases and regains the original values after 90 days. The nerve cell more than doubles its RNA content which reaches values of 470 $\mu\mu$g per cell. The RNA concentration is still low and reaches the original value after 70 days. The amounts of protein and lipid per cell are further increased and reach their maximal values concurrently with the RNA. The original value of the mass concentration is already restored to normal one month after the crush.

DISCUSSION

The magnitude of the changes in relation to time

Nerve regeneration implies a reaction in the proximal part involving a vigorous outgrowth of thin sproutings during the time of the so-called 'central degeneration' of the proximal nerve. The first sproutings occur within 3 hr of the trauma (PERRONCITO, 1907; RANSON, 1912; CAJAL, 1928) but these are abortive. The thin axons become organized during the outgrowth period, and they have penetrated the scar tissue by the fifth to sixth day. The period until the new axons appear in the peripheral part of the nerve is called the latent period. The axons then advance to the end plates with a velocity (for motor nerves) of around 4 mm per day (GUTMANN *et al.*, 1942). In our experiments the period of rapid outgrowth has been estimated as 12 days, following GUTMANN *et al.* The increase in size of the established axons occurs slowly during the maturation period, which lasts about 200 days (YOUNG, 1942).

According to the present experiments an average hypoglossal neuron replaces during the regeneration period, about 10^5 $\mu\mu$g of organic cell material i.e. fifty times

the amount of the material contained in the cell body. During the outgrowth period, however, the axons are thin, averaging around 0·5 μ in diameter, and the output per day will then average only 50 $\mu\mu$g.

During the latent period the only change observable in the cell body is the decrease in protein content, but the change towards a smaller particle size in the RNA also begins. The amount of RNA per cell remains constant during the outgrowth period, but the change in aggregation of the RNA becomes more marked during the rest of the period and gives the nerve cells the typical chromatolytic appearance, with a low absorbing capacity at 2600 Å. In three to five days the proteins and lipids of the cell body rapidly double in quantity and remain at this level with only a slight increase during the rest of the outgrowth period. The cell volume increases to 2·6 times the original volume and this depends mainly on a water uptake into the cell body.

Concomitantly with the sprouting of the axons in the first two days of the outgrowth period there is an increase of more than 100 per cent in the amount of proteins and lipids per cell. A 100 per cent increase in cell dry weight during such a short time means a vigorous protein production.

During the maturation period, which represents more than 95 per cent of the whole regeneration, the most conspicuous change is the increase in the amount of RNA per cell. The maximum occurs at 40 days with an average of 470 $\mu\mu$g per nerve cell, i.e. 160 per cent increase. The RNA concentration, however, goes slowly back to the normal values. The finely dispersed RNA in the central part of the cytoplasm in the fixed sections, observed during the outgrowth period, is now gradually replaced by the intensely absorbing aggregations corresponding to the usual Nissl picture. This happens during the first part of the maturation period.

The RNA increase observed, parallels an increase of a similar magnitude in the amount of protein and lipids per cell resulting in a doubled cell body weight. This increase in dry substance can be correlated with the volume increase. It is to be noted that the concentration of the cell material has returned to normal. Hence the volume increase observed is due to a real increase in mass. Contrasting with this the volume increase during the outgrowth period is mainly due to an increase in the water content of the cell. Volume, RNA, protein and lipids of the regenerating nerve cell return slowly to the starting points during the maturation period.

The high content of RNA in all types of nerve cells hitherto analysed which averages 150 to 1000 $\mu\mu$g RNA per cell and have a concentration of 0·5 to 3 per cent—or around 20 per cent RNA, computed on the dry protein basis—stresses an important characteristic of the nerve cell. It seems to be *the* RNA-containing somatic cell *par préférence*. The only type of cell that can compete seems to be the pancreatic cell of small animals with a high metabolism.

The extensive quantitative changes during regeneration of RNA and solid matter —protein and free lipid—indicates a high protein and RNA metabolism of the nerve cells. This conclusion has been drawn already on the basis of earlier microspectrographic studies on nerve cells during regeneration and various functional conditions (HYDÉN, 1943–1955). This hypothesis is strongly supported by the recent studies of GEIGER (1955) of the RNA production in the cerebral cortex after stimulation. At present we are pursuing the analysis of regenerating nerve cells using isotopes.

It seems not far-fetched to visualize the neuron as an enormous gland cell structure whose lively protein metabolism serves the specific nerve function.

VOL
1
1956/

Hypothesis concerning the nerve cell mechanism

The relation between RNA and protein increase. There is now good evidence that the RNA in a cell organism is a prerequisite for the formation of protein (ALLFREY, DALY and MIRSKY, 1953; GALE and FOLKES, 1954), although the DNA of the nucleus controls the protein formation on a long-term basis (BRACHET, 1954). This conclusion has been drawn from instances where an increase in the RNA amount can be correlated with a protein increase. During the maturation period this is exactly what we have found.

But our finding of a protein increase with constant RNA during the outgrowth period seems at variance with this view. The change in RNA aggregation from large particles to particles of submicroscopic size at a constant RNA level, however, presents a new feature of this problem. We would like to advance the working hypothesis that the change in RNA aggregation to small particulates means a transformation from an active to a more active form. This results in the synthesis of protein required for the production of the new axon.

Furthermore, when this distribution of RNA in the cell prevails, newly formed RNA occurs on the other side of the nuclear membrane and a gradient of the nucleoproteins has been shown from the nucleus to the nuclear membrane (HYDÉN, 1943). A nerve cell mechanism for the production of cytoplasmic nucleoproteins involving the activity of the nucleus and the cytoplasm simultaneously seems possible.

Previous studies on nerve regeneration using ultraviolet microspectrography (HYDÉN, 1941, 1943; GERSH and BODIAN, 1943) showed that the RNA concentration diminished in the chromatolytic area. This agrees well with the data now reported.

Analogy with nerve cells during embryonic development. It has frequently been pointed out that the chromatolytic nerve cells in regeneration bear a resemblance to embryonic nerve cells (BIERVLIET, 1900). Our data from the outgrowth period confirm this view. The water increase in the cell body, the change in configuration with constant amount of RNA and the rapid increase in proteins and lipids are indicative of a close correspondence between the regenerating and embryonic nerve cells studied earlier by our methods (SOURANDER, 1953; HYDÉN, 1953). Furthermore, the changes during the maturation period obtained in our study (aggregation of RNA and protein increase together with the volume increase) show a strong resemblence to the motor neurons during the late embryonic stage.

The role of re-establishment of function in the growth process. The nerve regeneration can also be considered as a problem of differentiation. This process in itself cannot at present be explored. Only by different types of criteria obtained from cytological, chemical and physiological studies the process of differentiation can be recognized. The primary problem, however, lies at the level of the gene action on the cytoplasm and the action of external or cell milieu factors back on the genes of the nucleus. As has been frequently pointed out the nucleus and the gene are surrounded by the cytoplasm from which raw material is provided to the genes. Since external conditions have been shown to affect the gene action, a change also in the internal cell milieu must be expected to get a detectable effect as a consequence of the gene action i.e. the equilibrium in the expression of the genes must be changed.

In nerve regeneration such a phenomenon seems to be observable. Our results show that a most conspicuous change occurs around the twelfth day in the regeneration. At this time there begins a second volume increase which is due to a high

increase in the amount of RNA, proteins and lipids. The volume increase during the outgrowth period on the other hand depends mostly on a water increase. The outgrowth period is finished when the thin axon has reached the periphery. This re-establishment of the functional contact is presumably the factor which changes the course of the chemical processes in the cell. The stimulus from the effector organ back to the cell probably represents the change in the internal cell milieu which permits the effect of another part of the genome to be expressed.

Especially in the nerve cell the nucleo-cytoplasmic relationship is important as is discussed on p. 323. If the relationship is disturbed, the nerve cell as a subject for differentiation will react on this change in the milieu with chemical and morphological changes. Seen from this aspect one might say that the effect of the gene activity is one when the thin axon rapidly grows out, and another during the maturation period. The re-establishment of the functional contact between neuron and effector organ signifies the starting point of the considerable production of nucleoproteins during the maturation period.

It has repeatedly been shown how important it is for the neuron during the embryonic development and in regeneration to achieve contact with the periphery in order to be fully differentiated chemically and morphologically (HAMBERGER and LEVI-MONTALCINI, 1950; HYDÉN, 1953; BARRON and MOTTET, 1953). If the functional stimulation is excluded, the growth process in the nerve cells is also inhibited (BRATTGÅRD, 1952).

All evidence shows that the neuronal function is of great importance for the highly differentiated neuron to develop chemically.

VOL
1
1956/

SUMMARY

(1) Nerve regeneration has been studied in the hypoglossal nucleus of the rabbit. Ultramicrochemical and quantitative cytochemical methods were used to determine the content per nerve cell of RNA and dry weight (divided into protein and lipid fractions). The data observed were correlated with observations on the peripheral growth of the axon.

(2) The weight of the fresh, control hypoglossal cell soma averages 2770 $\mu\mu$g (1 $\mu\mu$g $= 10^{-12}$ g). The amount of RNA per cell averages 200 $\mu\mu$g. The RNA concentration expressed as weight per volume is thus 1·5 per cent. Computed on a dry protein basis it averages 9 per cent. The volume of the cells averages 13,300 μ^3. During the period of outgrowth of the axons, the nerve cell changes were characterized by an increase in volume and in the lipid and protein content: there was also an uptake of water, causing a decrease in the concentration of the intracellular substance. The amount of RNA was constant during this period, but the state of aggregation changed, so that it formed small, finely dispersed particles. These changes form the chemical background of chromatolysis in nerve regeneration.

(3) The change in RNA aggregation during this period of rapid growth is interpreted as a transformation from an active to a more active form of RNA, serving as an initiator of the protein production which follows.

(4) The maturation period, which starts when contact is re-established between nerve cell and periphery, is characterized by another type of growth process. The most striking features are the 100 per cent increase in RNA content and the volume increase of 100 per cent. The latter is explained by the increase in the amount of

proteins and lipids per cell. The original state of aggregation of RNA is eventually restored, as are the original concentration values. The re-establishment of functional contact with the periphery is stressed as a factor of importance for the chemical reconstruction of the neuron.

Acknowledgements—Financial support for this investigation has been received from the Rockefeller Foundation, the Swedish Medical Research Council and from the Swedish Society for Cancer Research.

REFERENCES

VAN BIERVLIET J. (1900) *Le Névraxe* **33,** 1.
BODIAN D. and MELLORS R. C. (1945) *J. exp. Med.* **81,** 469.
BOURGHARDT S., BRATTGÅRD S., HYDÉN H., JIWERT B. and LARSSON S. (1953) *J. sci. Instrum.* **30,** 464.
BOURGHARDT S., HYDÉN H. and NYQVIST B. (1955) *Experientia* **11,** 163.
BRACHET J. (1954) *Recent Developments in Cell Physiology* (Ed. KITCHING) pp. 91–102. Butterworths London.
BRATTGÅRD S. O. (1952) *Acta Radiol.* Suppl. 96.
BRATTGÅRD S. O., EDSTRÖM J. E. and HYDÉN H. (1957) *Exp. Cell Res.* In press.
BRATTGÅRD S. O. and HYDÉN H. (1952) *Acta radiol.* Suppl. 94.
BRATTGÅRD S. O. and HYDÉN H. (1954) *International Revue of Cytology* (Ed. BOURNE and DANIELLI) Vol. III. pp. 455–474. Academic Press, New York.
CAJAL, RAMON y S. (1928) *Degeneration and Regeneration of the Nervous System* Humphrey Milford, London.
CAJAL, RAMON y S. (1952) *Histol. du Système Nerveux de l'Homme et des Vertebrés* Vol. I. Instituto Ramon y Cajal, Madrid.
CASPERSSON T. (1941) *Naturwissenschaften* **29,** 33.
EDSTRÖM J. E. (1953) *Biochim. biophys. Acta* **12,** 361.
EDSTRÖM J. E. (1957) *J. Neurochem.* In press.
ENGSTRÖM A. and LINDSTRÖM B. (1950) *Biochim. biophys. Acta* **4,** 351.
GALE E. F. and FOLKES J. P. (1954) *Nature, Lond.* **173,** 1223.
GEHUCHTEN A. VAN (1897) *La Cellule* **313,** 13.
GERSH I. and BODIAN D. (1943) *J. cell. comp. Physiol.* **253,** 21.
GUTMANN E., GUTTMAN L., MEDEWAR P. B. and YOUNG J. Z. (1942) *J. exp. Biol.* **14,** 19.
GUTMANN E. and SANDERS F. K. (1943) *J. Physiol.* **101,** 489.
HALLÉN O. (1956) *Acta anat.* Suppl. 25.
HAMBURGER V. and LEVI-MONTALCINI R. (1950) *Genetic Neurology* (Ed. WEISS) pp. 128–160. University of Chicago Press, Chicago.
HYDÉN H. (1943) *Acta Physiol.* Suppl. 17.
HYDÉN H. (1950) *Genetic Neurology* (Ed. WEISS) pp. 177–193. University of Chicago Press, Chicago.
HYDÉN H. (1953) *J. embr. exp. Morphol.* **1,** 315.
HYDÉN H. and LARSSON S. (1957) *J. Neurochem.* In press.
HÄGGQVIST G. (1937/38) *Anat. Anz.* Erg. B. 2. **85,** 191.
METTLER F. A. (1948) *Neuroanatomy* C. V. Mosby Comp., St. Louis.
MICKLEWRIGHT H. L., KURNICK N. B. and HODES R. (1953) *Exp. Cell. Res.* **4,** 151.
MOTTET K. and BARROW D. H. (1954) *Yale J. Biol.* **26,** 275.
NISSL F. (1892) *Allg. Z. Psychiat.* **48,** 197.
PERRONCITO A. (1907) *Beitr. path. Anat.* **42,** 354.
RANSON S. W. (1912) *J. comp. Neurol.* **22,** 487.
ROBINS E., SMITH D. E. and EYDT K. M. (1956) *J. Neurochem.* **1,** 54.
SOURANDER P. (1953) *Acta path. microbiol. scand.* Suppl. 95.
STOWELL R. E. (1941) *Stain Tech.* **16,** 67.
WEISS P. (1950) *Genetic Neurology* (Ed. WEISS) pp. 1–39. University of Chicago Press, Chicago.
WEISS P. (1953) *J. embr. exp. Morphol.* **1,** 181.
WEISS P. (1955) *Analyses of Development* (Ed. WILLIER, WEISS AND HAMBURGER) pp. 346–401. Saunders, Philadelphia.
YOUNG J. Z. (1942) *Physiol. Rev.* **318,** 22.

Journal of Neurochemistry, 1957, Vol. 1, pp. 326-333, Pergamon Press Ltd., London

5-HYDROXYTRYPTAMINE (SEROTONIN, ENTERAMINE) CONTENT OF STRUCTURES OF THE LIMBIC SYSTEM*

M. K. PAASONEN,† P. D. MACLEAN and N. J. GIARMAN
The Departments of Pharmacology and Physiology, Yale University School of Medicine, New Haven, Connecticut

(*Received* 4 *February* 1957)

IN PREVIOUS articles, evidence has been cited from bioelectric and behavioural studies that allows one to infer a dichotomy in the function of the phylogenetically old and new cortex (MACLEAN, 1954; MACLEAN *et al.*, 1955–56). This dichotomy has important implications because it bears upon the distinctive attributes of emotional and intellectual behaviour. The 'old' cortex comprises the so-called archicortex and mesocortex. The archicortex and the greater part of the mesocortex are contained in the great limbic lobe which is found in the brains of all mammals. The "old" cortex and its associated nuclear structures constitute a functionally integrated system which may be appropriately referred to as the limbic system (MACLEAN, 1952).

In a study of the effect of neuropharmacological agents on the bioelectrical activity of the brain it was found that, following the administration of reserpine, distinctive changes could be localized in the hippocampus (MACLEAN *et al.*, 1955–56), a structure which contains the greater part of the archicortex and which has important connections with the hypothalamus. Corresponding, but less pronounced, changes could also be localized in the region of the posterior hypothalamus. Subsequently, with the publication of the findings of PLETSCHER, SHORE, and BRODIE (1956), it became apparent that the evolution of the electroencephalographic changes (MACLEAN *et al.*, 1955–56) followed a time course strikingly similar to that of the depletion and restoration of 5-hydroxytryptamine (5-HT) in the brain which they found after administering large doses of reserpine to rabbits. Heretofore it has been claimed that the amount of 5-HT in the cerebral cortex is negligible compared with that in the hypothalamus (AMIN, CRAWFORD, and GADDUM, 1954; PAASONEN and VOGT, 1956). In view of the foregoing observations and the suggestion that 5-HT might be of neurophysiological importance (GADDUM, 1954; WOOLLEY and SHAW, 1954; BRODIE and SHORE, 1957), a study was made of the 5-HT content of the hippocampus and other cortical and subcortical structures of the limbic system. This communication reports the findings.

METHODS

Mongrel dogs of both sexes, weighing from 5·1 to 8·0 kg, were used. Three animals were treated with iproniazid,‡ an inhibitor of monoamine oxidase which is an enzyme catalysing the destruction of 5-HT. The drug was administered intraperitoneally in a dose of 70 mg/kg (as the base) and was given 4 hr before sacrifice, and 16 hr before, in the case of a third animal. All animals were sacrificed by exsanguination following light chloroform anaesthesia, and the brains were rapidly removed and

* Aided in part by a grant from the National Institutes of Health.

† Riker and Fulbright Fellow in Pharmacology from the University of Helsinki, Department of Pharmacology.

‡ Generously supplied to us by Hoffman-LaRoche, Inc., Nutley, New Jersey under the trade-name "Marsilid."

dissected. Immediately following the dissection, which was usually completed within half an hour, the 5-HT was extracted from the tissues by 95 per cent acetone according to the method of AMIN *et al.* (1954).

The amounts of 5-HT were estimated by the bioassay technique of TWAROG and PAGE (1953) which utilizes the isolated heart of *Venus mercenaria*. The heart was maintained at room temperature in an over-flow bath of 4·0 ml capacity. It was bathed in an artificial sea-water medium with the following composition in g/l.: NaCl, 23·0; KCl, 0·65; $CaCl_2$, 1·1; $MgCl_2 \cdot 6H_2O$, 5·0; and $NaHCO_3$, 0·2. This medium has been found suitable for the isolated hearts of a number of salt-water molluscs (GADDUM and PAASONEN, 1955). In order to prevent the cardio-inhibitory effects of any acetylcholine which might be present in the extracts, benzoquinonium chloride* (6 mg/l.) was always included in the bathing fluid. In most assays the standard 5-HT solution and the tissue extracts were added alternately at 2·5 min intervals and left in contact with the heart for one-half minute.

A number of studies on the biosynthesis of 5-HT by 5-hydroxytryptophane decarboxylase was made according to the method of GADDUM and GIARMAN (1956).

Basis for selection of tissue samples. The limbic lobe was so named by Broca because it surrounds (literally, forms a border around) the brain stem. This ring-like convolution is enveloped by two concentric bands of cortex that may be classified generally as archicortex and mesocortex. The mesocortex, which forms the outer band, makes up most of the superficial cortex of the limbic lobe. In evolution, the inner band of archicortex becomes largely buried through a process of folding, and in higher forms it undergoes so much displacement by the corpus callosum that the bulk of it comes to lie in the hippocampus in the infero-medial part of the temporal lobe. The samples of archicortical tissue were taken from the hippocampus. On the basis of cytoarchitectural and neuronographic studies the mesocortex can be subdivided into several areas. Anatomical landmarks served to guide the dissecting out of samples of the principal subdivisions. Specimens were taken from the subcallosal, anterior cingulate, posterior cingulate, and retrosplenial cortex, as well as from the anterior and posterior parts of the hippocampal gyrus, which contain, respectively, the so-called pyriform and entorhinal† cortex. From the standpoint of cytoarchitectural and functional considerations the perilimbic cortex bordering the rhinal fissure may be considered as part of the limbic system. Samples of such cortex were taken from the orbital gyrus.

The nuclear structures of the limbic system include the amygdala, septal nuclei, hypothalamus, anterior and medial nuclei of the thalamus, the habenula, and parts of the basal ganglia. Samples were obtained from those structures that allowed one to obtain a sufficient amount of tissue for the bioassay without encroaching on extralimbic structures. Accordingly, attention focused on the amygdala, septum, and hypothalamus.

For purpose of comparison and control, samples were taken from representative parts of the neocortex, as well as from a number of nuclear and other nervous structures not belonging to the limbic system (see Table 1), whose 5-HT content had been previously investigated by ourselves and others.

RESULTS

The average values of the amount of 5-hydroxytryptamine (as the free base) found in the various tissues, as well as the values obtained in individual experiments, are given in Table 1.

Cortex. In the cortical tissue studied, one finds that all the relatively high values were confined to a number of limbic structures. These included the medial pyriform cortex‡ (with the highest value), the hippocampus, and the entorhinal and lateral pyriform cortex. All the remaining limbic cortical structures, as well as the neocortical areas that were sampled, had considerably smaller amounts of the amine. To provide a ready comparison of the results of the assays with respect to anatomical relationships, the values for the superficial structures of the brain have been represented in Fig. 1,

* Generously supplied to us by the Sterling-Winthrop Research Institute, Rensselaer, New York, under the trade-name "Mytolon'.'

† Brodmann's term; Cajal referred to the corresponding cortex as the angular ganglion.

‡ Because of the contiguity of the medial pyriform cortex and the amygdala there is always the danger that in dissecting out one, part of the other might be included, allowance should be made for this possibility in appraising the results of assays of these structures.

depicting the medial and lateral surfaces of the dog's cerebrum. The figure shows an interesting finding on the distribution of 5-HT in the superficial limbic cortex. If one takes the region of the olfactory trigone as a starting point (identified in the figure as the space between the pyriform and subcallosal cortex) and makes a half-circle

TABLE 1.—5-HYDROXYTRYPTAMINE LEVELS (IN μmg/g) OF VARIOUS AREAS OF THE DOG'S BRAIN

	"Normal" Animals	Mean	Iproniazid treated animals	Mean
Limbic Cortex:				
Pyriform, medial	256; 560	408		
Pyriform, lateral	140; 279	179		
Hippocampus	203; 242; 335	260	410; 330	370
Entorhinal	138; 167; 234	180		
Subcallosal	82; 100; 112	100		
Orbital	84; 93	89		
Anterior Cingulate	18; 32; 37	29		
Posterior Cingulate	15; 16; 26	19		
Retrosplenial	<5; 18; 19	<19		
Limbic Nuclei and White Matter:				
Amygdala	286; 535; 645	489		
Septal Region	363; 557	460		
Hypothalamus	375	375	519; 555	537
	224–526(6)	370(P)*		
Region of Intercolumnar Tubercle	90; 217; 250	186		
Olfactory Bulb	78	78	125; 195	160
	25–62(3)	39(P)		
Fimbria	30; <50	<50		
Neocortex:				
Posterior Sigmoid|Sensory-	68; 102	85	51; 110	81
Anterior Sigmoid/ motor	13	13		
Occipital Polar (Visual 1)	10; 62; 75	36		
Ectosylvian (Auditory 1)	74	74		
Other Structures:				
Caudate	213; 245; 320	229	250; 343; 432	342
	88–480(6)	274(P)		
Region of Putamen	113; 116	115		
Cerebral White Matter	<4–<13(3)	0(A)†‡	22; 24	23
Sympathetic ganglia	19–<59(3)	0(P)	<21; <30	0

* (P) indicates data from PAASONEN and VOGT (1956).
† O indicates values below level of detection by the assay.
‡ (A) Indicates data from AMIN *et al.* (1954).

on the medial surface around the limbic lobe in either direction, one finds progressively lower values of 5-HT. The lowest values are found in the retrosplenial cortex, which represents approximately the half-way mark of the circle.

In view of the large size of the hippocampus, it was of interest to learn whether or not the distribution of 5-HT was uniform throughout its entire extent. In a number of experiments, therefore, the body of this elongated structure which is bounded at each end by the amygdala and fornix respectively, was divided into 2 or 4 approximately

equal segments for testing. The results of assaying these subdivisions are given in Table 2. The findings indicated no significant regional differences.

Table 2 shows, however, that when assays were performed on hippocampal tissue

TABLE 2.—5-HYDROXYTRYPTAMINE LEVELS (IN μmg/g) OF HIPPOCAMPUS *en masse* COMPARED WITH THOSE OF QUARTER- AND HALF-SEGMENTS

A. Hippocampus, inclusive of white matter

(1)	*S i* + *S ii* + *S iii* + *S iv* 203; 335			
(2)	*S i* + *S ii* 270		*S iii* + *S iv* 214	
(3)	*S i* 345(*r*) 230(*l*)	*S ii* 232(*r*) 262(*l*)	*S iii* 229(*r*) 250(*l*)	*S iv* 230(*r*) 250(*l*)

B. Hippocampus, exclusive of white matter

(1)	*S i* + *S ii* + *S iii* + *S iv* 102; 118; 158; 368	
(2)	*S i* + *S ii* 86; 94	*S iii* + *S iv* 56; 53

S i-S iv refer to consecutive quarter-segments of hippocampus, *S i* being proximal to amygdala, and *S iv* proximal to fornix. Whenever hippocampus was segmented, assays were made on all segments. *r* and *l* refer, respectively, to right and left hippocampus of the same animal.

exclusive of the white matter, one obtained values of 5-HT that were, in general, considerably lower than those of the whole hippocampus. This was unexpected because it has previously been found that 5-HT is only present in grey matter. Two experiments were performed to inquire into this discrepancy. The findings indicated that in experiments in which all white matter is carefully excluded, some grey matter rich in 5-HT and immediately contiguous with the white is sacrificed. In other words, it became evident that it is the grey matter immediately adjoining the white that accounts for maximum yields of 5-HT from the hippocampus. The white matter represented by the fimbria has a low value of the amine.

Nuclei. All the limbic nuclei that were tested—namely, the amygdala‡, septal, and hypothalamic—showed high values of 5-HT. They contained the amine in the same or higher quantities than the caudate nucleus. The considerably lower figure found in the putamen may, at least partly, be due to the difficulty of dissecting this area.

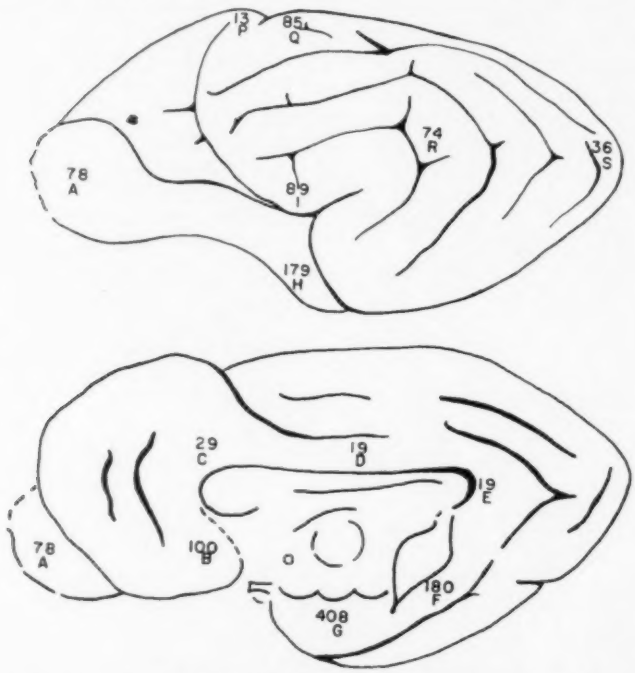


FIG. 1.—The upper representation is of the lateral surface of the dog's cerebrum, while the lower is of the medial surface (adapted from CAMPBELL, A. W., Histological Studies on the Localization of Cerebral Function, Cambridge University Press, 1905, p. 344). The numbers refer to μmg of serotonin per gram o tissue found in the various structures studied and labelled as follows:

Lower	*Upper*
A. Olfactory bulb	A. Olfactory bulb
B. Subcallosal	H. Lateral pyriform
C. Anterior cingulate	I. Orbital
D. Posterior cingulate	P. Anterior sigmoid
E. Retrosplenial	Q. Posterior sigmoid
F. Entorhinal	R. Ectosylvian (Auditory I)
G. Medial pyriform	S. Occipital polar (Visual I)

Region of intercolumnar tubercle. The intercolumnar tubercle derives its name from the fact that it lies between the columns of the fornix (PUTMAN, 1921). It therefore falls within the confines of the limbic system. WISLOCKI and LEDUC (1952) have pointed out that both in regard to cytology and to topographical relation to the tela chorioidea and subependymal plate, the tubercle appears to be the counterpart of the area postrema. As it had previously been reported that the area postrema has a high level of 5-HT, (AMIN *et al.*, 1954; PAASONEN and VOGT, 1956) it was of interest to learn whether or not the tubercle has a correspondingly high value. In order to obtain a sufficient amount of tissue for the test, the tubercle was removed and prepared along

‡ See the last footnote on page 327.

with a segment of the fornix. It is probable that this segment also contained tissue from the triangular nucleus of Cajal. The average value for three such samples was 186 μmg/g. PAASONEN and VOGT reported a value of 262 μmg/g for the area postrema.

Iproniazid. It was previously shown in this and other laboratories that the administration of iproniazid to rats leads to a marked increase of 5-HT in the brain. Hence, it became of interest to learn whether or not this drug would effect similar changes in various structures of the dog's brain. Three animals were used for this exploratory study. Vomiting, shivering, and other signs of malaise appeared 5 to 10 min following the administration of the drug. These effects seemed largely dissipated at the end of an hour. The values of the cerebral assays are given in Table 1, where they may be compared with those of untreated animals. Although the differences between treated and untreated animals did not appear as marked as we had been led to expect from the results in rats, the evidence suggests that iproniazid led to an increase in 5-HT in the caudate nucleus, olfactory bulb, hypothalamus, and hippocampus. In contrast,

TABLE 3.—RELATIVE 5-HYDROXYTRYPTOPHANE DECARBOXYLASE ACTIVITY OF REPRESENTATIVE NEURAL STRUCTURES

Tissue	Relative enzyme activity*
Sympathetic ganglia	10
Caudate nucleus	2
Hypothalamus	1
Hippocampus	1
Amygdala	0·5

* Based upon the conversion of 110 mg (10^{-4} M) 5-hydroxytryptophane to 1·8 mg 5-HT in 1 hr by the dog's hypothalamus. A value of 10 means 10 times this conversion.

and possibly because of the uneven distribution of monoamine oxidase in nervous structures (BOGDANSKI and UDENFRIEND, 1956), there appeared to be no significant change in regard to the posteriors igmoid gyrus ('sensory' cortex), or sympathetic ganglia, and the concentration in cerebral white matter was very low.

5-hydroxytryptophane decarboxylase activity. Table 3 summarizes the relative 5-hydroxytryptophane decarboxylase activity (5-HT synthesizing capacity) of a number of the structures under study. The results provide further evidence that, in general, nervous structures which contain stores of 5-HT are also able to synthesize it. There appears to be no consistent relationship, however, between the amount of 5-HT in a structure and its 5-HT-forming capacity.

DISCUSSION

On the basis of previous reports the impression is gained that the cerebral cortex generally contains meagre amounts of 5-HT compared with a number of subcortical structures. The present study has shown, however, that this amine is relatively abundant in parts of the cortex of the limbic lobe—specifically, the archicortex contained in the hippocampus and the mesocortex lying in propinquity to the olfactory tracts. It has also revealed that the two nuclei that are particularly linked with this cortex are as rich in 5-HT as the hypothalamus. Thus mutual participation of the septal

nuclei and amygdala with the hypothalamus in the regulation of autonomic function, may prove to correlate with the present findings.

Granted that its role in cerebral function is still not understood, it must be accepted as a significant fact that 5-HT is found in the brain and that it is present in greater concentration in some structures than in others. The value of descriptive data of the kind presented here lies in their ability to suggest further investigation that will help to elucidate the biological significance of this enigmatic substance. As yet, there is no convincing evidence that, as often suggested, it serves as a neural transmitter.

The finding that 5-HT is present in relatively large amounts in the hippocampus is of interest in light of the question raised in the introduction. This positive result still makes tenable the possibility that the electroencephalographic changes that can be localized to the hippocampus following a large dose of reserpine are in some way related to the profound effect of this drug on the metabolism of 5-HT. It would seem to give added significance to the observation that the time course of the appearance and disappearance of the EEG changes is roughly parallel to that of the depletion and restoration of 5-HT in the brain (see opening paragraph). Such a coincidence, however, cannot be taken to mean that 5-HT is implicated in brining about the EEG changes. The alterations in regard to both 5-HT and the EEG may be a reflection of other actions of reserpine on cerebral metabolism. There is indirect evidence, for example, from radioautographic studies employing ^{35}S-labelled L-methionine, that reserpine interferes with protein metabolism, and HOLZBAUER and VOGT (1956) have recently shown that it also releases norepinephrine from the brain. It is also pertinent to point out that, except for the extended time course, the electroencephalographic picture associated with reserpine has many similarities to that seen during the induction and recovery stages of ether anesthesia. According to PAASONEN and GIARMAN (1956) ether brings about no change in the levels of 5-HT in the brain.

VOL
1
1956/

SUMMARY

This report deals primarily with the results of a study of the 5-hydroxytryptamine (5-HT) content of cortical and subcortical structures of the limbic system. For the purpose of comparison and control, assays were performed on representative neocortical areas, as well as on a number of other non-limbic nervous structures.

All the cortical assays showing relatively high values of 5-HT were confined to limbic structures. These included the medial and lateral pyriform cortex, the entorhinal cortex, and hippocampus. All the remaining limbic cortical structures, as well as the neocortical areas that were sampled, had considerably smaller amounts of the amine. In the hippocampus (archicortex) the highest concentration of the amine was found to be in the gray matter immediately adjoining the white. Segments of the body of this structure showed no significant differences in assay values. In the limbic mesocortex there appeared to be a graded decline in values in a direction away from the olfactory trigone.

Assays of limbic nuclear structures were confined to the amygdala, septum, and hypothalamus. All had high values of 5-HT that were the same as, or exceeded, those of the caudate nucleus and of the tissue taken from the region of the putamen.

The region of the intercolumnar tubercle (located between the columns of the fornix which is the main conducting pathway of the hippocampus) had an average assay value which was more than three times that of the fimbrial fornix and was close

to that previously reported for the area postrema. The tubercle and the area postrema are recognized to have anatomical similarities.

The parenteral administration of a monoamine oxidase inhibitor, iproniazid, resulted in an increase in 5-HT in some structures, but not in others. A number of determinations of 5-hydroxytryptophane decarboxylase activity provided further evidence that nervous structures which contain 5-HT are generally able to synthesize it.

Acknowledgement—The authors wish to acknowledge the generous co-operation of Doctor V. L. Loosanoff, Director of the U.S. Fish and Wildlife-Service at Milford, Connecticut, who supplied the *Venus mercenaria* for this study.

REFERENCES

Amin A. H., Crawford T. B. B. and Gaddum J. H. (1954) *J. Physiol.* **126,** 596.
Bogdanski D. F. and Udenfriend S. (1955) *J. Pharmacol.* **116,** 7.
Brodie B. B. and Shore P. A. (1957) *Ann. N.Y. Acad. Sci.* **44,** 631.
Gaddum J. H. (1954) *Ciba Foundation Symposium* (*London*) p. 75.
Gaddum J. H. and Giarman N. J. (1956) *Brit. J. Pharmacol.* **11,** 88.
Gaddum J. H. and Paasonen M. K. (1955) *Brit. J. Pharmacol.* **10,** 474.
Holzbauer M. and Vogt M. (1956) *J. Neurochem.* **1,** 8.
MacLean P. D. (1952) *Electroenceph. clin. Neurophysiol.* **4,** 407.
MacLean P. D. (1954) *J. Neurosurg.* **11,** 29.
MacLean P. D., Flanigan S., Flynn J. P., Kim C., and Stevens J. R. (1955/6) *Yale J. Biol. Med.* **28,** 380.
Paasonen M. K. and Giarman N. J. (1956) Unpublished data.
Paasonen M. K. and Vogt M. (1956) *J. Physiol.* **131,** 617.
Pletscher A., Shore P. A. and Brodie B. B. (1955) *Science* **122,** 374.
Pletscher A., Shore P. A. and Brodie B. B. (1956) *J. Pharmacol.* **116,** 84.
Putnam T. J. (1922) *Johns Hopk. Hosp. Bull.* **33,** 181.
Twarog B. M. and Page I. H. (1953) *Amer. J. Physiol.* **175,** 157.
Wislocki G. B. and Leduc E. H. (1952) *J. comp. Neurol.* **96,** 371.
Woolley D. W. and Shaw E. A. (1954) *Proc. nat. Acad. Sci.* **40,** 228.

VOL. 1 56/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 334-339, Pergamon Press Ltd., London

ISOLATION OF FACTOR I

A. W. BAZEMORE,* K. A. C. ELLIOTT and E. FLOREY†
Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University, Montreal

(*Received* 11 *March* 1957)

INTRODUCTION

THE inhibitory activity of extracts of brain and spinal cord on the generation of impulses by the stretch receptor neuron of the crayfish has been reported previously (FLOREY, 1954). The agent (or agents) responsible for this action was called Factor I. In a previous communication in this Journal (ELLIOTT and FLOREY, 1956) the results of other published studies on Factor I were summarized, a method for the assay of Factor I activity was described and the effects of a number of known substances on the stretch receptor preparation were noted.

In the study now reported the assay method has been applied in the isolation from beef brain of a substance with high Factor I activity. This substance has been identified as γ-aminobutyric acid.

EXPERIMENTAL

Assay and units. Following every step in the purification, samples of the products were held for assay. The assay of Factor I activity was carried out as described by ELLIOTT and FLOREY (1956). Briefly this method consists in the application of the unknown material, freed from organic solvent and dissolved in a buffered saline solution, to the crayfish stretch receptor preparation. Various dilutions of the material are tested until a concentration, the "minimum blocking concentration," is reached which just blocks the spontaneous discharges of the stretch receptor neuron for 10 sec. Since the sensitivity of test preparations varies, the minimum blocking concentration of a reference solution containing Factor I is determined before and after each unknown. A single reference solution was used throughout the present work; samples were taken as required from the stock reference solution which was stored in the frozen state. The activity of the unknown solution was thus compared with that of the reference solution and the concentration of Factor I was expressed in 'c.u.r.' (crayfish units reference) per ml. A concentration of 1 c.u.r. per ml is about the minimum blocking concentration with many stretch receptor preparations.

Table 1 summarizes the results of the steps in the isolation procedure described below. It should be understood that the objective was to obtain increased specific activity (activity per unit weight of material) rather than maximal yields of activity.

Extraction of beef brain. Fresh beef brains, 52 kg, were minced and transferred to a glass-lined kettle containing 450 l. of acetone. After stirring for 15 min, the mixture was allowed to settle and then filtered through a canvas filter on a ceramic filter pot. The filter cake was washed with 68 l. of acetone and pressed dry on the filter with the aid of rubber dam. The filtrate was discarded.

The moist filter cake was returned to the kettle and to it were added 270 l. of '70% alcohol,' a mixture consisting of 20 vol. of water and 80 vol. of ethanol containing 10% methanol. After

* Present address: Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey, U.S.A.
† Present address: Department of Zoology, University of Washington, Seattle, Washington, U.S.A.

VOL
1
1956/

TABLE 1.—SUMMARY OF THE RESULTS OF PURIFICATION PROCEDURES

	Weight of solid	Total activity Thousands of c.u.r.	Specific activity c.u.r./mg
Acetone dried brain 450 l.	8600 g	3000	0·35
Acetone extract	—	76	—
70% Alcohol extract (4400 ml)	1260 g	2200	1·7
70% Alcohol residue	7300 g	200	0·03
70% Alcohol extract (3600 ml)	1060 g	1800	1·7
Acetone-pet. ether extracts	—	18	—
Acetone-pet. ether residue	508 g	850	1·7
95% Ethanol extract	25·2 g	400	16
Hot 95% ethanol extract	21·6 g	120	6
Hot 95% ethanol residue*	120 g	530	4·4±
95% Ethanol extract†	25·2 g	270	11
IRC–50 Effluent	18·4 g	260	14
IR–45 Effluent	14·7 g	320	22
IR–120 Effluent	—	<2·8	—
IR–120 First 3 l. of NH_3 eluate	2·9 g	360	124
IR–120 Next 2 l. of NH_3 eluate	260 mg	<3	10
IR–120 First eluate, after evaporation	2·9 g	470	160
IRA–410 Effluent and wash	—	1·5	—
IRA–410 First NH_3 eluate, 3 l.	114 mg	25	220
IRA–410 Second NH_3 eluate, 3 l.	570 mg‡	200‡	350
IRA–410 Third NH_3 eluate, 1 l.	446 mg	50	112
IRA–410 Second eluate concentrated	570 mg	200	350
Dowex–50: Effluent	—	<0·5	—
Pre-eluate, 3 l.	—	<3	—
1st eluate, 100 ml	—	1·4	—
2nd eluate, 100 ml	430 mg	93	220
3rd eluate, 100 ml	230 mg	67	290
4th eluate, 100 ml	—	1·2	—
5th eluate, 100 ml	—	0·5	—
6th eluate, 1500 ml	—	<7·5	—
Dowex–50 eluates 2 and 3	660 mg	160	240
Ethanol-extracted crystals	420 mg	150	350
Crude crystals	100 mg	35	350
Prisms	18 mg	8·6	480
γ-Aminobutyric acid, synthetic			500

* Assay uncertain due to the presence of stimulatory material.

† This extract was the same as the 95% ethanol extract listed above. Variations in assay sometimes occurred

‡ Some lost.

stirring overnight, the mixture was filtered. The filter cake was washed with 45 l. of 70% ethanol, pressed dry on the filter, and then discarded. The filtrate, which contained most of the original activity, was evaporated at 20°C to 4·4 l.

Further solvent treatments. The following solvent treatments were applied mainly in order to remove, by petroleum ether extraction, lipid materials which had been found to interfere with the subsequent use of ion exchange resins.

Of the above concentrate, 3·6 l. were added to 32 l. of acetone with stirring. The mixture was allowed to settle and the supernatant fluid was decanted. To the gummy precipitate, 6 l. of petroleum ether (b.p. 30–60°) were added. After vigorous stirring, the mixture was allowed to settle and the petroleum ether was removed by decantation and finally by centrifugation and decantation. After several such washes with petroleum ether, the residue was washed with acetone, filtered, and placed in a desiccator for several days.

To this pasty residue (508 g) which still contained some petroleum ether, 1·5 l. of 95% ethanol were added. After several stirrings, the mixture was allowed to stand overnight at room temperature. The supernatant fluid was decanted, and the extraction was twice repeated. On standing in the refrigerator, the ethanol extracts deposited fatty materials which were inactive. The three extracts were combined, evaporated to about 100 ml. and poured into 1 l. of petroleum ether. The lower layer was drawn off, evaporated to dryness, and taken up in 105 ml of water. This extract contained about half of the activity of the petroleum ether-washed cake. The ethanol-extracted residue was stirred with 2 l. of 95% ethanol and refluxed for 15 min. After cooling and filtering the hot extraction of the residue was repeated with 1 l. of 95% ethanol. These two extracts contained some additional activity, but a large part of the activity remained in the residue. The assay of this residue was complicated by the presence of stimulatory material.

Purification with ion exchange resins. A column was prepared containing approximately 100 g of washed Amberlite IRC–50 (a weak, carboxylic acid resin) in the acid form. The 105 ml of petroleum ether-extracted 95% ethanol-extract mentioned above were passed through this column followed by water. Most of the active material passed directly through the column although the washes continued to show activity until a litre of washings had been collected.

As they were collected, the effluent and the washes from the IRC–50 column were passed through a column containing 100 g of washed Amberlite IR–45 (a weak basic amine resin) in the basic form. A final wash was made with 500 ml of water. The active material passed directly through the column with no evidence of hold-up on the column.

The effluents from the IR–45 column were passed, in the succession in which they were collected, through a column containing 100 g of washed Amberlite IR–120 (a strong, sulphonic acid resin) in the acid form. The resin was washed with 4 l. of water. The combined effluents and washes showed little or no activity. The resin was then eluted with 3 l. of 0·5N-NH_4OH. (An additional 2 l. of 0·5N-NH_4OH eluate was collected but contained little solid matter and had no activity.) The first 3 l. of eluate, which contained all the activity, were evaporated to 160 ml in order to remove the ammonia.

This concentrated eluate was passed through a column containing 100 g washed Amberlite IR–410 (a strongly basic, quaternary ammonium resin) in the basic form. The resin was washed with 3 l. of water. Only a small amount of activity appeared in the effluent. The column was eluted with 0·5N-NH_4OH. After 1 l. of eluate had been collected the active material began to come off the column. The eluates were evaporated to dryness to remove the ammonia and then taken up in water. An unfortunate loss of a portion of the second eluate was responsible for low recovery at this stage. The remainder of the second eluate was taken up in 180 ml of water.

This latter solution was passed through a column containing 100 g of 8 per cent cross-linked, 50–100 mesh, washed Dowex-50 (a strong, sulphonic acid resin) in the acid form. The resin was then washed with 1 l. of water. The effluent showed no activity. The resin was eluted with 0·1N-NH_4OH. After 3 l. had passed through the column the eluate became alkaline and the active material came off the column in a very narrow band.

Crystallization and identification. When the second and third eluates from the Dowex-50 column were evaporated *in vacuo* to dryness, the residues crystallized in the evaporating flasks. Ethanol (95 per cent) was added in an attempt to redissolve the material, but it was not very soluble in the ethanol. The material was collected on a filter, the two fractions being combined in this operation. After drying in a desiccator over calcium chloride, the crystals weighed 420 mg.

VOL
1
1956/

Of this crystalline material, 100 mg were dissolved in about 200 ml of boiling 95% ethanol by repeated extraction with small portions. After filtering, no crystals formed on standing, on seeding and scratching, on cooling, or even on standing in the deep freeze chamber. The solution was evaporated to 50 ml when crystallization just began. Small bead-like crystals formed first; these were filtered off and found inactive. The filtrate was allowed to stand open to the atmosphere in a small beaker. As evaporation continued, other crystals formed which when examined under a tenfold magnification were found to be rock-like prisms with a rhomboid structure. The mother liquor was decanted, the crystals were washed with about 200 ml of 95% ethanol, collected and dried over calcium chloride. These crystals exhibited very strong inhibitory activity on the crayfish stretch receptor neuron. They weighed 18 mg and melted at 193–201°C after softening at 181°C.

By examination of the infrared spectrum of the prisms, they were identified as γ-aminobutyric acid. A paper chromatogram of the crude crystalline material (butanol: acetic acid: water, 4:1:5 v/v) had located the active material at R_F 0·26 in the centre of a large ninhydrin-positive spot. A comparative paper chromatogram of synthetic γ-aminobutyric acid and the prisms showed that the activity of the former centered at about R_F 0·24, the latter at R_F 0·26, both in ninhydrin-positive spots. Other ninhydrin-active spots which were given by solutions of the crude crystals (from R_F 0·1–0·4) had virtually disappeared in the chromatogram of the prisms, although there was still some scattering over that of the synthetic γ-aminobutyric acid chromatogram.

Recrystallization from acetone-water of the first crude crystals from the Dowex-50 eluates gave at least two types of crystals. One of these types, needle clusters, was strongly active; it could be γ-aminobutyric acid but not enough was obtained for identification.

Authentic γ-aminobutyric acid (m.p. 189–191°) synthesized by MERCK & Co. Inc. showed the same activity as the isolated material.*

Of forty-five other amino acids and other compounds of physiological interest tested, obvious inhibitory activity on the stretch receptor was observed† only with γ-amino-β-hydroxybutyric acid‡ which was about half as active as γ-aminobutyric acid, with β-alanine and δ-aminovaleric acid which were about one-twentieth as active and with α,γ-diaminobutyric acid which was about one-sixtieth as active. Carnitine and β-hydroxybutyric acid seemed inactive.

DISCUSSION

The presence of γ-aminobutyric acid in brain was first reported by AWAPARA, LANDURA, FUERST and SEALE (1950), ROBERTS and FRANKEL (1950) and UDENFRIEND (1950). It is present in brain extracts in the free state though, in native brain, most of it is in an "occult" form, either loosely bound or occluded by some membrane (ELLIOTT and FLOREY, 1956). Various authors have reported the presence of γ-aminobutyric acid in micro-organisms and plants but, in animal tissues, its concentration is high apparently only in brain (TALLAN, MOORE and STEIN, 1954); grey matter contains more than white and there is none in peripheral nerve (ROBERTS, HARMAN and FRANKEL, 1951). The Factor I activity in mixed beef brain is equivalent to about 150 μg of γ-aminobutyric acid per gram of brain. ELLIOTT and FLOREY (1956) found about 100 c.u.r. per g of fresh rat cerebral hemispheres, which corresponds to about 200 μg per g. ROBERTS *et al.* (1951) found about 400 μg of γ-aminobutyric acid per g of

* After the studies with the resins had indicated that the active substance might be an amino acid but no activity had been found in any α-amino acid tested, a commercial sample of material labelled γ-aminobutryic acid had been tested by the assay method and found to be inactive. This sample was later found not to be γ-aminobutyric acid.

† Tests of several of these substances were made by Mr. N. VAN GELDER in this laboratory, and by Dr. MAX BEN and Mr. J. DE GRAW at Merck Sharp and Dohme Research Laboratories.

‡ We are grateful to Professor T. HAYASHI for supplying us with a sample of γ-amino-β-hydroxybutyric acid.

adult mouse brain and TALLAN *et al.* (1954) found 240 μg per g of cat brain. There thus seems to be more than sufficient γ-aminobutyric acid present in brain to account for the whole Factor I activity found. Quantitative comparisons of Factor I activity with the chemically determined amount of γ-aminobutyric acid in the same sample of tissue are in progress.

Until recently γ-aminobutyric acid has been considered mainly from the point of view of its role in intermediary metabolism. ROBERTS and FRANKEL (1950) have shown that it is produced in brain by the decarboxylation of glutamic acid by a glutamic acid decarboxylase which requires pyridoxal phosphate as co-factor. BESSMAN, ROSSEN and LAYNE (1953) and ROBERTS and BREGOFF (1953) have shown that it undergoes a transamination reaction with α-ketoglutarate to produce glutamic acid and succinic semialdehyde. The latter substance may enter the tricarboxylic acid cycle and thus be utilized for energy production.

The studies of FLOREY and MCLENNAN (1955, 1956) and FLOREY (1956) have indicated that Factor I strongly affects the activity of various nervous structures. The present study shows that γ-aminobutyric acid has a marked effect on the activity of a neuron in the crayfish. Results of experiments to be published shortly by K. IWAMA and H. JASPER, from this Institute, have shown marked effects of topically applied γ-aminobutyric acid on electrical responses in the brain. KILLAM (1957) and BAIN and KILLAM (1957) have shown that hydrazide intoxication is accompanied by a fall in the γ-aminobutyric acid level of the mammalian brain concomitant with the convulsive activity produced by the hydrazides. HAYASHI and NAGAI (1956) have reported that γ-amino-β-hydroxybutyric acid applied to the cortex or injected into the carotid artery or the cerebrospinal fluid inhibits electrically or chemically induced convulsions in dogs. (This substance is not known to be present in brain.) γ-Aminobutyric acid was also inhibitory in low concentration but, in higher concentration, it became a "stimulant".

It thus seems probable that γ-aminobutyric acid is concerned in some aspects of the regulation of physiological activity in the brain and other nervous structures. It is possibly a transmitter substance of inhibitory impulses, being released from the occult form when certain inhibitory neurons discharge. Alternatively, a concentration of the free substance, varying with conditions, may be maintained in the tissue fluids and exert a moderating effect on neuronal activity.

The mechanism of action of γ-aminobutyric acid remains to be elucidated. It is likely that it causes a hyperpolarization of the relevant neuronal membrane. Its structure has features in common with that of acetylcholine and this may be the basis of its activity (FLOREY, 1956).

SUMMARY

A crystalline material showing Factor I activity (inhibition of the spontaneous discharges of the crayfish stretch receptor neuron) has been isolated from beef brain and has been identified as γ-aminobutyric acid. A synthetic sample of γ-aminobutyric acid shows the same Factor I activity. γ-Amino-β-hydroxybutyric acid showed about half the activity of γ-aminobutyric acid. β-Alanine, δ-aminovaleric acid and α,γ-diaminobutyric acid were active in considerably higher concentrations.

Acknowledgements—This work was aided by grants from Merck & Co. Inc., Rahway, New Jersey. The beef brains were kindly supplied by Canada Packers Ltd., Montreal, Quebec. The large-scale

VOL.
1
1956/5

extraction was carried out with the facilities of Merck and Co. Ltd., Valleyfield, Quebec. Infrared analyses were carried out by Mr. ROBERT WALKER of Merck & Co., Inc., Rahway, New Jersey.

We are grateful for all the above-mentioned co-operation and assistance and wish to acknowledge especially our appreciation of the advice and encouragement of Dr. NORMAN BRINK of Merck & Co., Inc.

REFERENCES

AWAPARA J., LANDAU A. J., FUERST R., and SEALE B. (1950) *J. biol. Chem.* **187,** 35.
BESSMAN S. P., ROSSEN J. and LAYNE E. C. (1953) *J. biol. Chem.* **201,** 385.
ELLIOTT K. A. C. and FLOREY E. (1956) *J. Neurochem.* **1,** 181.
FLOREY E. (1954) *Arch. int. Physiol.* **62,** 33.
FLOREY E. (1956) *Canad. J. Biochem. Physiol.* **34,** 669.
FLOREY E. and MCLENNAN H. (1955, 1956) *J. Physiol.* **129,** 384; **130,** 446.
HAYASHI T. and NAGAI K. (1956) *Abstracts of Communications XX Internat. Physiol. Congress* p. 410.
KILLAM K. F. (1957) *J. Pharmacol.* **119,** 263.
KILLAM K. F. and BAIN J. A. (1957) *J. Pharmacol.* **119,** 255.
ROBERTS E. and BREGOFF H. M. (1953) *J. biol. Chem.* **201,** 393.
ROBERTS E. and FRANKEL S. (1950) *J. biol. Chem.* **187,** 55.
ROBERTS E., HARMAN P. J. and FRANKEL S. (1951) *Proc. Soc. exp. Biol. Med.* **78,** 799.
TALLAN H. H., MOORE S. and STEIN W. H. (1954) *J. biol. Chem.* **211,** 927.
UDENFRIEND S. (1950) *J. biol. Chem.* **187,** 65.

OL.
1
6/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 340-347, Pergamon Press Ltd., London

THE EFFECT OF NERVE SECTION ON THE FREE AMINO ACIDS OF NERVOUS TISSUE

G. PORCELLATI* and R. H. S. THOMPSON
Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.1

(*Received* 4 *April* 1957)

VOL
1
1956/

THERE exist in the literature a number of reports on the content of various free amino acids in brain tissue, but, apart from the work of LEWIS (1952; 1954) on the free amino acids of invertebrate nerve, we have been unable to find any quantitative account of the levels of different amino acids in vertebrate peripheral nerve, or of any changes that these levels may undergo during the Wallerian degeneration that follows nerve transection.

Since, in the past, we have used hen sciatic nerve for observations on other changes following transection (CAVANAGH, THOMPSON and WEBSTER, 1954; CAVANAGH and WEBSTER, 1955), we have again used hen tissues in the present work. To satisfy ourselves as to the adequacy of our technical methods, we began the present study using rat brain, for which a number of published values for the levels of free amino acids are available. We next studied the levels in hen brain, hen spinal cord and hen sciatic nerve, and finally the changes that these levels undergo in the sciatic nerve at various times after transection of the nerve.

The extraction of the free amino acids from the nerve tissue was carried out according to the method of ROBERTS and FRANKEL (1950). After separation by paper chromatography, and detection with ninhydrin, the individual amino acids were eluted and estimated quantitatively by a modification of the method of CLARKSON and KENCH (1956).

EXPERIMENTAL

Materials. Wistar rats of either sex, weighing between 150 and 250 g, and fully grown hens of mixed breed, weighing about 2 kg, were used.

In transection experiments the right sciatic nerve was cut high up under nembutal and ether anaesthesia as previously described (CAVANAGH *et al.*, 1954), in each case care being taken to prevent any re-innervation of the distal stump.

The animals were killed by decapitation, and the tissues for investigation quickly removed and frozen at $-15°$. The whole brain was used, and the whole spinal cord from the upper cervical to lower lumbar levels; in the case of the sciatic nerve, corresponding lengths of the right and left nerves were dissected out from just below the point of emergence of the nerve from the greater sciatic notch, or from the point of transection in the case of sectioned nerves, down to approximately the level of the knee.

Preparation of tissue extracts. After freezing, the tissue (brain or spinal cord) was weighed and dispersed in a Potter-Elvehjem homogenizer in 13 vol. of 75% ethanol. In the case of the sciatic nerves, the frozen tissue was ground in a small mortar, 13 vol. of 75% ethanol being gradually added with further grinding until only a small residue of undispersed fibrous tissue remained.

The homogenates were then centrifuged at 3000 rev/min for 15 min. The almost clear supernatants so obtained were transferred to glass evaporating basins, and evaporated almost to dryness on a water-bath at temperatures not exceeding 50°.

* British Council Scholar, University of Perugia, Italy.

The yellowish residue was taken up in a measured volume of distilled water (varying between 0·7 ml and 10 ml according to the weight of tissue used and the particular tissue being investigated), transferred to a centrifuge tube, and centrifuged at 7500 rev/min for 45 min to remove suspended insoluble matter. By this means clear extracts were obtained, which were almost colourless when derived from spinal cord or sciatic nerve, but were faintly yellow in the case of brain. These extracts were used directly for chromatography, without preliminary de-salting.

Chromatographic procedure. The volume (100–150 μl) of the extract used for chromatography was usually equivalent to 50 mg of fresh tissue, although a volume equivalent to 100 mg was applied when we were studying those amino acids (leucine, valine, and tyrosine) which are present only in low concentrations and of which the R_F values indicated good resolution from other compounds.

All the amino acids and related compounds which we have estimated were identified on the chromatograms by the use of markers and by their stability, or instability as in the case of glutamine, to acid hydrolysis (2 hr at 100° in 6 N-HCl). Whatman No. 1 paper, washed for 24 hr in running water, and dried at room temperature, was used throughout, and two-dimensional chromatograms were made, using first *n*-propanol-water 4:1 (v/v) and then phenol-water 4:1 (w/w). With the phenol-water mixture, NaCN (0·1 g) was placed in a beaker in the tank to prevent oxidation of the phenol (DENT, 1948). The solvent was allowed to ascend for 18 hr in the case of propanol, and 16 hr in the case of phenol. At the end of the second run (in phenol) the papers were hung in air at room temperature for 1 hr, after which they were immersed for 15 min in anhydrous ether, this immersion being repeated 3–4 times with fresh ether. The papers were then finally dried in air at room temperature.

In some experiments an alternative solvent, devised by Dr. R. W. R. BAKER in this laboratory, was used in place of propanol; this was methyl ethyl ketone—methyl cellosolve—20% acetic acid, 40 : 15 : 30 (v/v/v).

Quantitative estimation of amino acids. Ninhydrin-reacting materials were detected on the chromatograms by dipping the papers in a ninhydrin-$SnCl_2$ reagent prepared by the following modification of the method of CLARKSON and KENCH (1956).

$SnCl_2,2H_2O$ (Hopkin & Williams Ltd. A.R.) (8 mg) are dissolved in 10 ml 0·1 M-citrate buffer, pH 5·0. Ninhydrin (B.D.H., A.R.) (0·5 g) is added, and the mixture shaken for a few minutes, made up to 100 ml with *iso*propanol, and shaken again for 30 min. This solution was prepared freshly for each experiment.

After dipping, the excess ninhydrin reagent was allowed to drain from the paper, which was then placed in a hot-air oven at 50° for 20 min. Under these conditions the background remained uncoloured. 1–2 hr after colour development the ninhydrin spots, identified by the preliminary use of markers, together with a blank piece of paper, were cut out. In each case the area of paper cut out for each spot and for the blank was the same. The pieces were then shredded finely with scissors, and placed in glass-stoppered test-tubes. Aqueous ethanol (4 ml of 50 per cent) was added, and the tubes were shaken mechanically for 20 min. The contents of the tubes were then filtered under pressure (nitrogen) through sintered glass filters. The optical density of the filtrate was measured at 570 mμ in a spectrophotometer against 50 per cent aqueous ethanol.

A standard solution of appropriate amounts of each amino acid under investigation was prepared in 100 ml H_2O, to which 2–3 drops of conc. HCl had been added. For each experiment 5 μl of this solution were applied to a paper, and run in the two solvents. The paper was then treated as described above, and each spot cut out, eluted and estimated together with the corresponding spot derived from the paper on which the tissue extract had been run.

RESULTS

As mentioned earlier, the initial experiments in this study were carried out with rat brain. Under our conditions we were able to identify the following ninhydrin-reacting compounds: glutathione, aspartic acid, glutamic acid, serine, glycine, taurine, threonine, tyrosine, alanine, glutamine, γ-aminobutyric acid, valine, leucine, phenylalanine, phosphorylethanolamine and phosphorylserine. Apart from a provisional identification by ROBERTS (1956), free phosphorylserine has not, as far as we are aware, been definitely identified in tissue extracts; because of this, the evidence of identification of this compound—R_F value, P content, effect of acid hydrolysis,

and behaviour on an ion-exchange column—is being described in another paper (PORCELLATI, 1957). Phosphorylserine and phenylalanine were present in concentrations so low as to render them often undetectable on inspection of the chromatograms. In addition, 4 other ninhydrin-reacting spots were seen, but were not identified. From the observed high R_F values of two of these spots in phenol, and from their slow movement in propanol, it is thought that these may have been arginine and histidine respectively, but owing to their poor resolution, particularly in propanol, it was decided not to proceed further with their examination.

TABLE 1.—CONCENTRATIONS OF CERTAIN FREE AMINO ACIDS IN RAT BRAIN; A COMPARISON WITH PUBLISHED VALUES
(5 rat brains studied; levels expressed as μg amino N/g wet wt. of brain)

	Mean level	Range	Published values
Aspartic acid	45·1	42·5–49·5	38[g], 44[f], 50·7[e].
Glutamic acid	131·5	120·4–138·0	150[d], 132[g], 165[f], 129[e], 145[h].
Glutamine	59·8	56·4–65·2	71[d], 35·3[f], 55[h].
γ-Aminobutyric acid	81·0	77·5–86·0	86[g], 28[g], 48[f].
Alanine	13·5	12·8–14·8	8·0[g].
Taurine	36·0	30·2–40·0	75[g], 33·1[e].
Serine	13·5	11·6–15·4	15[g].
Glycine	21·2	18·5–24·4	18[g].
Valine	3·0	2·5–3·5	2·2[b], 1·7[c].
Leucine	2·1	1·8–2·4	2·1[b], 1·0[c].
Tyrosine	1·9	1·6–2·2	1·3[b], 1·6[c].
Threonine	7·9	6·9–8·7	15·5[b], 12·0[c].

[a] AWAPARA, LANDUA, FUERST and SEALE (1950).
[b] WILLIAMS, SCHURR and ELVEHJEM (1950).
[c] SCHURR, THOMPSON, HENDERSON, WILLIAMS and ELVEHJEM (1950).
[d] DAWSON (1950).
[e] AWAPARA, LANDUA and FUERST (1950).
[f] CRAVIOTO, MASSIEU and IZQUIERDO (1951).
[g] ANSELL and RICHTER (1954).
[h] SCHWERIN, BESSMAN and WAELSCH (1950).

Since at the start of this study we were interested primarily in the free amino acids in nervous tissue, we have estimated only the free amino acids listed above, together with taurine but with the exception of phenylalanine, for each of which we obtained good separation chromatographically under our conditions. The mean values for the concentrations of these compounds found in the brains of 5 rats are given in Table 1. In this Table we have included the levels reported by earlier workers. Although in some cases there are discrepancies in the published values, it will be seen that the concentrations of each amino acid which we have found agree with one or more of the earlier results.

As a further check on the techniques which we were using we next carried out some recovery experiments with pure amino acids. To test the adequacy of our chromatographic, staining and elution procedures, measured samples of solutions of pure amino acids were placed on paper, and cut out before and after running in the solvents; these were eluted and estimated as described above. The amino acids

VOL
1
1956/

used in these experiments were glutamine, glycine, threonine and alanine, and in each case 95–105 per cent recovery was obtained after chromatography. Further, to test the adequacy of our extraction technique, known amounts of pure amino acids were added to the tissue homogenates, and the extraction and estimation carried out as already described; recoveries of 86–102 per cent were obtained.

The results of the experiments with hen tissues are summarized in Table 2.

TABLE 2.—FREE AMINO ACIDS OF HEN BRAIN, SPINAL CORD AND SCIATIC NERVE

(Mean values ± S.E.; expressed as μg amino N/g wet wt. of tissue)

	Brain (5)	Spinal cord (11)	Sciatic nerve (22)
Aspartic acid	53·3	44·8 ± 1·42	15·7 ± 0·64
Glutamic acid	137·6	95·7 ± 2·38	23·6 ± 0·69
Glutamine	51·8	40·0 ± 0·88	13·8 ± 0·72
γ-Aminobutyric acid	84·0	12·3 ± 0·24	2·1*
Alanine	14·7	14·6 ± 0·56	6·1 ± 0·32
Taurine	13·6	11·8 ± 0·95	7·3 ± 0·33
Serine	14·8	11·4 ± 0·77	4·8 ± 0·22
Glycine	24·6	17·0 ± 0·42	7·8 ± 0·35
Valine	3·6	3·2 ± 0·16	2·1 ± 0·08
Leucine	2·7	2·5 ± 0·16	1·9 ± 0·09
Tyrosine	1·9	2·0 ± 0·09	—†
Threonine	7·1	3·7 ± 0·22	2·8 ± 0·14

(Numbers in brackets = number of animals used)

* Not always found; value given represents mean of 4 experiments in which this amino acid was detected.

† Present only in trace amounts, or not found.

The levels of free amino acids in hen brain agree closely with those found in rat brain, with the exception of taurine, which is present in a lower concentration in the avian tissue. In the spinal cord, although alanine, valine, leucine and tyrosine are present in as large amounts as in brain, the other amino acids estimated are present in smaller amounts, this applying particularly to γ-aminobutyric acid. In the sciatic nerve the levels of all the free amino acids studied are lower than in the spinal cord. This again is particularly apparent for γ-aminobutyric acid, which is present in such small amounts in sciatic nerve as to have rendered it undetected in 18 out of 22 experiments. The other ninhydrin-reacting compounds, which we found in brain but which we did not identify, are also present in extracts of spinal cord and sciatic nerve.

In these two latter tissues, however, a further spot was observed which was not present in brain, or at most only in traces, and which showed R_F values of 0·25 in phenol and 0·21 in the methyl ethyl ketone solvent mixture, while hardly moving in propanol. When hydrolysed, the compound giving rise to this spot yielded about six amino compounds and appeared therefore to be peptide in nature. The properties and composition of this compound are now being investigated.

In the experiments on the effect of nerve section, extracts were prepared from the

TABLE 3.—FREE AMINO ACIDS CONTENT OF HEN SCIATIC NERVE AT INTERVALS AFTER NERVE SECTION

(Expressed as μg amino N/g wet wt. of nerve; mean of 2 experiments for each time after nerve section.)

	Controls*	Days after section						
		4	8	13	15	18	23	28
Aspartic acid	15·7	13·3	13·9	12·1	*23·8*	20·0	12·7	14·1
Glutamic acid	23·6	30·9	33·4	39·3	*54·7*	42·4	30·4	28·2
Glutamine	13·8	13·6	13·4	19·8	*27·2*	17·1	17·1	19·8
Alanine	6·1	8·8	10·4	21·5	*21·8*	13·2	12·0	15·1
Taurine	7·3	5·3	10·4	*17·3*	15·8	13·6	9·0	9·0
Serine	4·8	5.2	10·1	*17·0*	15·9	11·3	9·2	9·7
Glycine	7·8	6·2	15·5	11·8	*21·8*	15·1	12·1	11·0
Valine	2·1	2·5	5·0	*6·0*	*6·0*	4·0	2·7	4·5
Leucine	1·9	2·6	4·8	*5·6*	5·0	4·5	2·1	3·7
Threonine	2·8	2·9	5·6	6·2	*13·1*	8·1	5·0	4·8

* Mean of 22 birds.

sectioned sciatic nerve and from the contralateral normal nerve of the same animal at intervals of from 4 to 28 days after transection, 2 hens being killed at each of the days studied. In Table 3 the mean values for the levels of free amino acids in the sectioned nerves are given; the controls given in this Table represent the mean of 22 normal birds.

The values for the sectioned nerves are expressed in terms of the wet weight of the nerve, and no allowance is made in this Table for the increase in the water content of the nerve which occurs after transection; this point will be discussed later. It will be seen that the levels of each of the amino acids studied underwent a considerable increase after transection, maximum values being reached at about 13–15 days from operation. Owing to the differences in the levels of amino acids in the normal nerves

TABLE 4.—CHANGES IN CONTENT OF FREE AMINO ACIDS OF HEN SCIATIC NERVE AFTER TRANSECTION

(Results expressed as percentage change in sectioned nerve, compared with level in normal nerve of same bird; average of 2 experiments for each day after transection.)

	Days after transection						
	4	8	13	15	18	23	28
Aspartic acid	−21	−20	+15	+*65*	+25	−16	−28
Glutamic acid	+15	+39	+97	+*143*	+58	+20	+20
Glutamine	+5	+44	+62	+72	+28	−5	+2
Alanine	+39	+89	+220	+*257*	+116	+117	+138
Taurine	+11	+58	+123	+*137*	+54	+23	+21
Serine	+18	+76	+*336*	+196	+156	+73	+54
Glycine	−7	+66	+138	+*165*	+97	+43	+26
Valine	+3	+86	+*186*	+176	+94	+58	+69
Leucine	+10	+131	+*242*	+211	+94	+43	+45
Threonine	+14	+77	+281	+*328*	+142	+52	+57

VOL 1 1956/

of healthy birds, it is of interest also to see the percentage change in the sectioned nerve compared with the normal nerve of the same animal (Table 4). From this Table it is apparent that aspartic acid and glutamine undergo the smallest percentage changes of any of the amino acids studied, while serine and threonine exhibit the largest percentage increases.

γ-Aminobutyric acid and tyrosine were also present in increased amounts in the sectioned nerves, but, owing to the faintness of the spots caused by these acids, quantitative estimation was not attempted; in some of the experiments tyrosine was not detected in the normal nerve, although a definite, if faint, spot was visible in the sectioned nerve.

DISCUSSION

The results obtained with hen tissues in the course of this work indicate the striking similarity in the content of different free amino acids in hen brain and in rat brain; such similarity had already been noticed for cat, rat, mouse and pigeon brain, with regard to their glutamic acid and glutamine content by KREBS, EGGLESTON and HEMS (1949) and by SCHWERIN, BESSMAN and WAELSCH (1950). Of the compounds studied by us, taurine is the only one that shows a significant difference, rat brain containing nearly 3 times as much as hen brain.

It will be noticed also that only small variations were found in the levels of these amino acids in the 5 rat brains studied. Further, the values that we have obtained for this tissue agree well with other published values for rat brain. CRAVIOTO, MASSIEU, and IZQUIERDO (1951) obtained lower values for glutamine than those found by us, but it should be pointed out that the method used by these workers involved a tenfold concentration of their extracts by boiling, a procedure which would cause a partial conversion of glutamine to pyrrolidonecarboxylic acid and ammonia; in addition, these workers, and also ANSELL and RICHTER (1954), obtained lower values for γ-aminobutyric acid.

The levels of free amino acids in the spinal cord are in most cases only slightly lower than in brain, although γ-aminobutyric acid is an exception in that spinal cord contains only about 1/7 of the concentration present in brain. With the exception of valine and leucine, the levels of the other free amino acids studied in the sciatic nerve are, however, considerably less than in brain, γ-aminobutyric acid being present in only 1/40 of the amount present in brain. ROBERTS, FRANKEL and HARMAN (1950) have already reported that, while mouse and rabbit brain and spinal cord contain relatively large amounts of this amino acid, they were unable to detect it in rabbit sciatic nerve. The generally low levels of free amino acids found in sciatic nerve can probably be related to the admixture of the truly neuronal tissue with the relatively large amounts of connective tissue in the peripheral nerves.

In the case of each of the 10 amino acids studied, section of the sciatic nerve caused a considerable increase, the maximum level being reached 13–15 days following transection. The results of these experiments have been expressed as μg amino N/g wet weight of nerve, without making any allowance for the considerable increase in wet weight, due to oedema, which is known to occur in degenerating nerve (JOHNSON, MCNABB, and ROSSITER, 1949; CAVANAGH *et al.*, 1954). Since the compounds that we are here interested in are of low molecular weight, and are present also to some extent in extracellular fluid, we have thought it misleading to attempt to allow quantitatively for this increase in water content, although if we multiply our results

by the ratio of the wet weight of the degenerating nerve to that of a similar length of normal nerve from the opposite leg, the increases which we have recorded would be higher still.

We have at present insufficient evidence to put forward any detailed hypothesis concerning the mechanism responsible for this increase in the amounts of free amino acids present in degenerating peripheral nerve. It is known of course that brain contains a number of proteolytic enzymes; thus, KIES and SCHWIMMER (1942) have described an active cathepsin, ANSELL and RICHTER (1954) a proteinase active at pH 7·4, ABDERHALDEN and CESAR (1940) a polypeptidase, and POPE and ANFINSEN (1948) a dipeptidase. Although we are not aware that work on these enzymes has been extended to peripheral nerve it would seem very probable that this general increase in free amino acids in degenerating nerve may be due to an acceleration of the proteolysis that may normally be concerned with the turnover of cell and myelin proteins in the healthy nerve. As already pointed out, the results in Table 4 show that serine and threonine exhibit the largest percentage increases among the amino acids studied; it is of interest in this connection that LOGAN, MANNELL and ROSSITER (1952) reported a fall in phosphoprotein phosphorus in degenerating cat sciatic nerve, particularly since it now seems that in another phosphoprotein, bovine casein, the phosphorus is bound not only to serine (LIPMANN, 1933), but also to threonine (DE VERDIER, 1952). There is of course the further possibility that part at any rate of the increase in serine is due to its release from phosphatidyl serine which, according to BRANTE (1949), is one of the myelin lipids. In order to investigate this possibility further we are now making a study of the levels of phosphorylated compounds, such as phosphorylserine, phosphorylethanolamine and phosphorylcholine, in brain and in degenerating nerve.

VOL
1
1956/

SUMMARY

(1) A quantitative study has been made of the levels of a number of free amino acids in rat brain, hen brain, hen spinal cord and hen sciatic nerve, in the latter case using both normal nerve and nerve at intervals during degeneration after transection.

(2) The following ninhydrin-reacting compounds have been detected and identified in these nervous tissues; aspartic acid, glutamic acid, glutamine, serine, glycine, taurine, threonine, tyrosine, alanine, γ-aminobutyric acid, valine, leucine, phenylalanine, glutathione, phosphorylserine and phosphorylethanolamine. Four other ninhydrin-reacting spots were regularly seen, but were not identified.

(3) The levels of each of the 10 amino acids studied in degenerating sciatic nerve were found to undergo a considerable increase, the maximum level being reached 13–15 days following transection. Serine and threonine were found to show the largest percentage increases.

Acknowledgements—We wish to thank the British Council for a Scholarship to one of us (G. P.), and the Medical Research Council for a grant towards the expenses of this work. Our thanks are also due to Dr. G. R. WEBSTER for his assistance in the experiments involving nerve section.

REFERENCES

ABDERHALDEN E. and CESAR G. (1940) *Fermentforschung* **16,** 255.
ANSELL G. B. and RICHTER D. (1954) *Biochem. J.* **57,** 70.
ANSELL G. B. and RICHTER D. (1954) *Biochim. biophys. Acta* **13,** 92.
AWAPARA J., LANDUA A. J. and FUERST R. (1950) *Biochim. biophys. Acta* **5,** 457.

AWAPARA J., LANDUA A. J., FUERST R. and SEALE B. (1950) *J. biol. Chem.* **187,** 35.
BRANTE G. (1949) *Acta physiol. scand.* (*Supplement* 63) **18,** 1.
CAVANAGH J. B., THOMPSON R. H. S. and WEBSTER G. R. (1954) *Quart. J. exp. Physiol.* **39,** 185.
CAVANAGH J. B. and WEBSTER G. R. (1955) *Quart. J. exp. Physiol.* **40,** 12.
CLARKSON T. W. and KENCH J. E. (1956) *Biochem. J.* **62,** 361.
CRAVIOTO R. O., MASSIEU G., and IZQUIERDO J. J. (1951) *Proc. Soc. exp. Biol. N.Y.* **78,** 856.
DAWSON R. M. C. (1950) *Biochem. J.* **47,** 386.
DENT C. E. (1948) *Biochem. J.* **43,** 169.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1949) *Biochem. J.* **45,** 500.
KIES M. W. and SCHWIMMER J. (1942) *J. biol. Chem.* **145,** 685.
KREBS H. A., EGGLESTON L. V. and HEMS R. (1949) *Biochem. J.* **44,** 159.
LEWIS P. R. (1952) *Biochem. J.* **52,** 330.
LEWIS P. R. (1954) *J. Physiol.* **126,** 34P.
LIPMANN F. (1933) *Biochem. Z.* **262,** 3.
LOGAN J. E., MANNELL W. A. and ROSSITER R. J. (1952) *Biochem. J.* **51,** 482.
POPE A. and ANFINSEN C. B. (1948) *J. biol. Chem.* **173,** 305.
PORCELLATI G. (1957) *J. Neurochem.* In press.
ROBERTS E. (1956) *Neurochemistry* (Edited by KOREY S. R. and NURNBERGER J. I.) p. 11. Cassell, London.
ROBERTS E. and FRANKEL S. (1950) *J. biol. Chem.* **187,** 55.
ROBERTS E. FRANKEL S., and HARMAN P. J. (1950) *Proc. Soc. exp. Biol. N.Y.* **74,** 383.
SCHURR P. E., THOMPSON H. T., HENDERSON L. M., WILLIAMS J. N. and ELVEHJEM C. A. (1950) *J. biol. Chem.* **182,** 39.
SCHWERIN P., BESSMAN S. P. and WAELSCH H. (1950) *J. biol. Chem.* **184,** 37.
DE VERDIER C.-H. (1952) *Nature, Lond.* **170,** 804.
WILLIAMS J. N., SCHURR P. E. and ELVEHJEM C. A. (1950) *J. biol. Chem.* **182,** 55.

VOL.
1
56/57

Journal of Neurochemistry, 1957, Vol. 1, pp. 348–357 Pergamon Press Ltd., London.

EXCITANTS AND DEPRESSANTS OF THE CENTRAL NERVOUS SYSTEM, ON ISOLATED ELECTRICALLY-STIMULATED CEREBRAL TISSUES

H. McIlwain and Olga Greengard
From the Department of Biochemistry, Institute of Psychiatry
(British Post-Graduate Medical Federation, University of London)
Maudsley Hospital, London, England

(*Received* 27 *March* 1957)

VOL
1
1956/

Present observations concern substances with marked effects on the brain *in vivo:* butobarbitone, a general depressant, and methylethylglutarimide (megimide) which antagonizes its action; chlorpromazine, mepazine and reserpine which have more specific depressant actions; and pentamethylenetetrazole (leptazole) and a thiobarbiturate which are convulsants. How the majority of these substances have their central effects is largely unknown and the present account concerns one stage only in an analysis of mode of action: namely, attempts to see whether relevant effects can be reproduced in isolated cerebral tissues at concentrations of the drugs corresponding to those active *in vivo*.

A logical stage in such analysis would be to examine the effects of adding the substances to chemically defined fluids perfusing the isolated mammalian brain, but maintenance of cerebral activities under such circumstances has not yet been achieved (Geiger and Yamasaki, 1956). Isolated cerebral tissues in chemically defined fluids can however respond metabolically to applied electrical pulses (McIlwain, 1955; 1956a) and modification of such response by an added drug is here used as an indication that the drug is acting on the tissue. It is to be emphasized that the metabolic effect measured is taken only as an index of the tissue's response to electrical pulses and that when a drug has metabolic sequelae in such a system, direct action of the drug on the metabolic process measured is not implied. Response has been judged in the present study by measurement of respiration and glycolysis (for other measurements see McIlwain, 1955; Greengard and McIlwain, 1955), and as necessary controls, observations have been made of the effects of the substances studied, on respiration and glycolysis in absence of pulses as well as in their presence. Observations on yohimbine and hydrallazine are also included.

EXPERIMENTAL

Materials and methods followed closely those of an earlier study of anticonvulsants (Greengard and McIlwain, 1955), especially with regard to salines, application of electrical pulses, arrangement of electrodes and tissues, and the manner of interpreting and expressing results. Variations are noted below; in addition, anaerobic effects of pulses were studied under the conditions of McIlwain (1956b) in bicarbonate salines. We gratefully acknowledge supply of the following materials: butobarbitone (5:5-ethylbutylbarbituric acid) and thiogardenal (2-thio-5:5-ethylphenylbarbituric acid) from Dr. L. Buchel, Institut Fournier, Paris; reserpine and hydrallazine from Ciba Laboratories; chlorpromazine from May & Baker Ltd; mepazine from William R. Warner & Co. and megimide (2:2-methylethylglutarimide) from Dr. T. A. B. Harris, Guy's Hospital Medical School, London.

Dr. BUCHEL (private communication) reported the intravenous convulsive dose of thiogardenal in rats as 75 mg/kg, the animals recovering after some hours; 50 mg/kg gave slight muscular tremors and 100 mg/kg were fatal.

Chlorpromazine was determined according to DUBOST and PASCAL (1953). Reserpine was examined as a suspension made by grinding the solid in the medium of the experiment in a test-tube homogenizer.

RESULTS

Excitants

Previous examination (ANGUIANO and MCILWAIN, 1951) showed that pentamethylenetetrazole, picrotoxin, strychnine and caffeine had little or no effect on respiration and certain other metabolic characteristics of isolated cerebral tissues. Present control experiments (Table 1) showed pentamethylenetetrazole to be without

TABLE 1.—EXCITANTS ON RESPIRATORY AND GLYCOLYTIC RESPONSES OF GUINEA PIG CEREBRAL CORTEX

Pulses	Respiration (μmoles O_2/g/hr) with:		Lactic acid formed (μmoles/g/expt.) with:	
	No addition	Drug (1 mM)	No addition	Drug (1 mM)
None	60 ± 3 (6)	P: 59 ± 3 (6)	22 ± 3 (6)	23 ± 4 (6)
Condenser, 3 v, 100/sec	76 (3)	P: 79 (3)	—	—
Condenser, 5 v, 100/sec	81 (3)	P: 84 (3)*	—	—
Condenser, 10 v, 2/sec	69 (3)	P: 70 (3)*	—	—
Sine wave, 50 cyc., 0·3 v	64 ± 3 (10)	P: 71 ± 3 (10)	28 ± 2 (10)	P: 26 ± 3 (10)
Sine wave, 50 cyc., 0·5 v	76 ± 3 (7)	P: 80 ± 3 (10)	34 ± 3 (7)	P: 30 ± 1 (7)
Sine wave, 10 cyc., 1 v	74 ± 2 (12)	P: 79 ± 2 (12)	28 ± 3 (7)	P: 36 ± 4 (6)
Sine wave, 2000 cyc., 2 v	70 ± 3 (6)	P: 76 ± 4 (6)	32 ± 2 (10)	P: 33 ± 4 (10)
None	63 ± 3 (6)	T: 60 ± 4 (6)	—	—
Sine wave, 10 cyc., 1 v	72 ± 5 (6)	T: 74 ± 6 (6)	—	—
Sine wave, 50 cyc., 1·5 v	117 (3)	T: 114 (3)	—	—
Sine wave, 2000 cyc., 2 v	69 (4)	T: 66 (4)	—	—
ANAEROBIC EXPERIMENTS				
None	—	—	89 (2)	P: 90 (2)
Sine wave, 2000 cyc. 3 v	—	—	78 (4)	P: 74 (4)

Experiments were aerobic except in the instances noted.

Drugs: P, pentamethylenetetrazole; T, thiogardenal. These were added to the experimental salines before beginning the experiment, except in the instances asterisked in which the drugs were tipped from sidearms to give the concentration quoted. Condenser pulses were always alternating, of the peak voltages quoted and of time-constant 0·4 msec. When 2 pulses were given/sec, these were separated by a 10 msec. interval. Sine wave alternating current is described in terms of cyc./sec. and virtual voltage. Results are usually followed by standard deviations, and by the number of values (in parentheses).

effect also on the tissue's glycolysis; a convulsant barbiturate, thiogardenal, was also without effect on respiration.

In judging whether these agents affected the electrically excited tissue, the intensity of applied electrical pulses in the majority of experiments was deliberately limited to values which, in the absence of added drug, gave only about one quarter of the

tissue's maximal metabolic response. As pulses at greater intensity could bring about a much larger response (see Table 1 under 50 cyc/sec sine-wave current), much scope thus remained for action in an excitant agent. Nevertheless, little or no action was found (Table 1) in the drugs added. Possible increases or decreases in the effects of brief condenser pulses of exponential time-voltage relationships were examined; these pulses were arranged to give suboptimal response either by application at low voltage or infrequently. Under the latter conditions, protoveratrine can potentiate response (Wollenberger, 1955; McIlwain, unpublished). Interaction of the convulsants was also sought with sine-wave currents arranged to give suboptimal response through application at low voltage or at high or low frequencies. No interaction was found (Table 1); anticonvulsants affect especially the sequelae of high-frequency sine-wave stimulation (Greengard and McIlwain, 1955). In most of the present experiments, the convulsants were present in the fluid in which the tissue was immersed, from the commencement of the experiment; thus some 40 min were allowed for equilibration between drug and tissue before pulses were applied. However, in the experiments marked with asterisks in Table 1, pulses were applied to half the tissues throughout the experiments and the drug added to the fluid during the incubation. In no cases were progressive or other changes seen which could be ascribed to the drugs. Depressant effects could also have been seen in these experiments, (and were sought using pulses which gave greater response), but were not observed.

Electrical pulses applied to cerebral tissues under anaerobic conditions, which were otherwise similar to those of the preceding experiments, caused decreased glycolysis (McIlwain, 1956*b*). Pentamethylenetetrazole did not alter this action of the pulses. In addition to the determination of lactic acid quoted in Table 1, the course of formation of the acid was followed during these and other experiments, by manometric measurement of carbon dioxide evolved from bicarbonate-glucose-salines but no difference was caused by the drug.

A barbiturate and antagonist

Interactions between butobarbitone and 2:2-methylethylglutarimide were sought; *in vivo* the imide rouses the subject from barbiturate depression (Shaw, Simon, Cass, Shulman, Anstee and Nelson, 1954). *In vitro*, the imide alone had little effect on respiration or glycolysis of cerebral tissue until high concentrations were reached (Fig. 1), when it acted in the same fashion as did butobarbitone, in decreasing respiration and increasing glycolysis. Mixtures of the two substances had roughly additive effects. Antagonism was not found under these circumstances, in spite of the examination of a considerable range of concentrations of each compound. The concentrations of each compound which showed metabolic effects under the conditions of Fig. 1, were much greater than those at which their actions as depressant and antagonist are shown *in vivo*.

Experiments with electrically stimulated cerebral cortex were accordingly carried out largely with low concentrations of the barbiturate and antagonist, concentrations which had themselves little or no action on the tissue in absence of pulses. Thus 0·3 mM-butobarbitone prevented the greater part of the respiratory and glycolytic response of the tissue to pulses (Table 2; see McIlwain, 1953) but this action was largely unaffected by the imide. In reaching this conclusion, stimulation of the tissue's metabolism was carried out with the variety of pulse-types indicated in Table

VOL
1
1956/

2, and was examined with different ratios of barbiturate and imide. On the stimulated metabolism of the tissue also, the action of the two substances is seen on some occasions to be additive, especially when higher concentrations of the imide were employed, but not to be antagonistic.

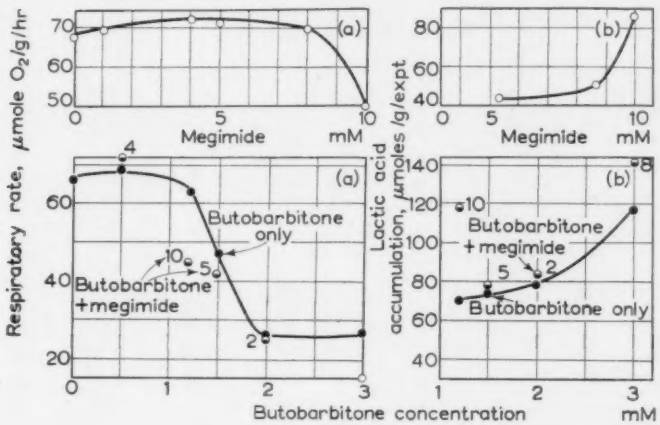


FIG. 1.—(a), respiration, and (b), formation of lactic acid, by unstimulated guinea pig cerebral cortex in the presence of butobarbitone and of $\beta\beta$-methylethylglutarimide (megimide). When both substances are present (◒) the concentration of the imide is given in mM by figures beside the points.

Phenothiazines

Chlorpromazine has been used therapeutically at a wide range of dose levels between 1 and 30 mg/kg. In experimental animals, between 2 and 25 mg/kg produced increasing central effects in potentiating the depressant effect of ether and in causing hypothermia in mice, rats and guinea pigs (COURVOISIER, FOURNEL, DUCROT, KOLSKY and KOETSCHET, 1953). At 2 mg/kg in dogs DUBOST and PASCAL (1953) could not detect the substance in the blood but at 20 mg/kg levels of about 20 μM were observed. In the present experiments major effects were found to be caused by 10 μM-chlorpromazine (Fig. 2).

Respiration and glycolysis of cerebral tissues in absence of applied pulses, were relatively insensitive to chlorpromazine. In agreement with COURVOISIER *et al.* (1953) a small, delayed inhibition only was given by 500 μM solutions. The greater sensitivity reported by GANSHIRT and BRILMAYER (1954) was not observed. However, applied electrical impulses rendered the tissue very much more sensitive to the drug (Table 3). Respiratory response to pulses was unaffected by 3 μM-chlorpromazine but increasingly depressed by levels of 5 to 20 μM. Similar sensitivity was shown in metabolic response to a variety of pulse types: to sine-wave currents and to condenser pulses applied intermittently or continuously and at a variety of voltages (Fig. 2). Glycolytic response to electrical pulses was also inhibited by comparable levels of the drug.

Chlorpromazine was found to be assimilated into cerebral tissues during experiments of the type described. During their course, approximately half the chlorpromazine added to reaction mixtures at 20 μM left the fluid for the tissue, there reaching levels of 0·5 to 1 μmole/g. This maximum level was reached after 15 to 30 min

TABLE 2.—BUTOBARBITONE AND METHYLETHYLGLUTARIMIDE ON RESPONSES OF GUINEA PIG CEREBRAL CORTEX

Pulses	Substances added (mM)		Respiration (% increase above control)	Lactic acid formation (μmoles/g/exp)
	Butobarbitone	Megimide		
Condenser, 7 v, 0·1 msec	0	0	62	72
	0·3	0	6	56
	0	0·5	20	53
	0·3	0·5	8	56
Condenser, 7 v, 0·4 msec	0	0	70	72
	0·5	0	20	88
	0	1	60	80
	0·5	1	6	78
Condenser, 10 v, 0·1 msec	0	0	69	75
	0·5	0	21 ± 6 (10)	73 ± 8 (10)
	0	4	50	65
	0·5	4	20 ± 4 (10)	80 ± 6 (10)
Condenser, 10 v, 0·4 msec	0	0	80	77
	1	0	22	75
	0	8	54	99
	1	8	18	112
Sine-wave, 0·5 v, 2000 cyc.	0	0	45	40
	0·5	0	18 ± 4 (8)	50 ± 2 (8)
	0	0·5	59	44
	0·5	0·5	14 ± 3 (8)	47 ± 2 (8)
	0	5	39	64
	0·5	5	0	47

Experiments were aerobic, with the drugs present in phosphate glucose saline throughout the experiments. Pulses were applied during the second of three consecutive 35-min periods during which respiration was measured. Other details, as described in Table 1.

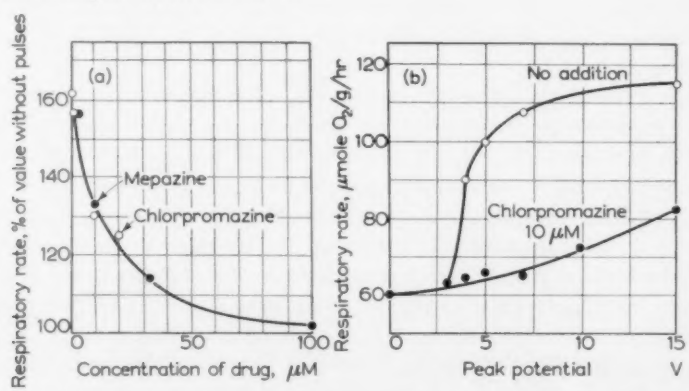


FIG. 2.—Inhibition by phenothiazines of respiratory response by guinea pig cerebral cortex to electrical pulses. (a). Effects on tissue stimulated by alternating condenser pulses of peak potential 10 v, time-constant 0·4 msec, applied at 100/sec. (b). Response of tissue in presence and absence of chlorpromazine, to pulses of the duration and frequency just quoted, but varying in voltage.

TABLE 3.—PHENOTHIAZINES, RESERPINE AND HYDRALLAZINE ON RESPIRATORY AND GLYCOLYTIC RESPONSES OF GUINEA PIG CEREBRAL TISSUES

Pulses	Substances added (μM)	Change from controls without drug or pulses (%)	
		Respiration	Lactic acid accumulation
0	Chlorpromazine, 10, 20	0	0
Condenser, 7 v, 0·4 msec	0	73	32
Condenser, 7 v, 0·4 msec	Chlorpromazine, 10	6	16
Condenser, 10 v, 0·4 msec	0	80	106
Condenser, 10 v, 0·4 msec	Chlorpromazine, 10	15	45
Condenser, 10 v, 0·4 msec	Mepazine, 10	28	18
Condenser, 10 v, 0·4 msec	Mepazine, 100	2	4
0	Mepazine, 10, 30, 100	0	0
Condenser, 7 v, 0·4 msec	0	27	—
(2 pulses each 0·15 sec)	Chlorpromazine, 5	19	—
Condenser, 10 v, 0·4 msec	0	46	—
(2 pulses each 0·25 sec)	Chlorpromazine, 5	31	—
	Chlorpromazine, 10	16	—
Sine-wave, 3·5 v, 2000 cyc.	0	41	—
	Chlorpromazine, 5	18	
	Chlorpromazine, 10	13	
0	Reserpine	0	0
Condenser, 5 v, 0·4 msec	0, or reserpine	20	—
Condenser, 10 v, 0·4 msec	0, or reserpine	88	—
Condenser, 15 v, 0·4 msec	0, or reserpine (midbrain)	96	78
Condenser, 15 v, 0·4 msec	0, or reserpine (thalamus)	98	72
0	Hydrallazine, 1000	−11	−26
0	Hydrallazine, 100, 10	0	0

Experiments were with guinea pig cerebral cortex except in the instances noted. Pulses are characterized as described in Table 1, and were applied to grid electrodes containing a tissue slice except in the experiments with midbrain and thalamus, when the mechanically chopped, cell-containing tissue was floating freely in vessels with concentric electrodes. Reserpine was added at 0·2 μmoles/ml, but was only partly in solution; all substances were present in saline with tissue from the commencement of experiments; pulses were applied after 30 to 40 min. incubation. Values quoted are averages from at least 3 experiments which agreed to within 15 (usually 10) per cent.

incubation of slices of 35 to 70 mg, in 3·5 ml fluid. In the experiments of Table 3 and Fig. 2, electrical pulses were normally applied to the tissue after 30 to 40 min incubation: that is, at a time when equilibrium had been reached between tissue and solution. The concentrations found in the tissue *in vitro* are only a little higher than those of 0·3 μmoles/g found by BERTI and CIMA (1954) in the brain of mice which had received 100 mg/kg of chlorpromazine subcutaneously.

Mepazine, a phenothiazine differing in structure from chlorpromazine but similar in its type of pharmacological action (NIESCHULZ, POPENDIKER and SACK, 1954), was also examined in the present experiments. Its effects on isolated cerebral tissues proved closely similar to those of chlorpromazine. Respiration and glycolysis were

again little affected by mepazine until stimulated by applied pulses, when levels of 10 μM and higher inhibited both types of metabolic response (Table 3, Fig. 2).

Reserpine, hydrallazine and yohimbine

Reserpine, having clinical applications similar to certain of those of chlorpromazine, was examined in the fashion just described in the case of chlorpromazine. Reserpine was however without effect on respiration and glycolysis of cerebral cortex both before and during the application of the different pulse-types of Table 3. It was also without effect on different metabolic sequelae of the pulses, namely, the fall in respiration and glycolysis induced by applying pulses in absence of glucose. Further, as reserpine is suspected to act on parts of the brain other than the cerebral cortex, action was sought on tissues from the midbrain and thalamus of the guinea pig. Glycolytic and respiratory responses to pulses in tissues from these parts were similar to those observed in the cerebral cortex, and again no action of reserpine was detected.

Metabolic effects of hydrallazine, which like reserpine exerts hypotensive effects possibly in part by central action, were also sought. In particular it has been thought that, as a substituted guanidine, hydrallazine might like other guanidines release aerobic glycolysis (DICKENS, 1939; GREENGARD and MCILWAIN, 1955). This was not however the case; and 1 mM-hydrallazine slightly inhibited both respiration and glycolysis of cerebral tissues while lower levels were without effect, in a phosphate-glucose saline (Table 3), in a bicarbonate-glucose saline, and also in the presence of high concentration of potassium salts (30 mM) in either medium.

Yohimbine among previously known alkaloids is closest in structure to reserpine; it also has been the subject of therapeutic trials in mental illness. Present investigations showed electrically stimulated cerebral tissues to be very sensitive to the compound (Fig. 3). Levels of 8 μM, which were without effect on the respiration of the tissue in absence of pulses, halved the tissue's respiratory response to applied pulses. Higher levels of yohimbine had limited effect in depressing respiration in absence of pulses, but still showed a much greater proportional effect in their presence. Response to a range of stimulating voltages was similarly inhibited (Fig. 3*B*).

DISCUSSION

In their response to chlorpromazine, mepazine, and yohimbine, electrically stimulated cerebral tissues thus represent the most sensitive *in vitro* system yet discovered. For other actions of these substances *in vitro*, higher concentrations have been required. Thus respiratory processes in preparations from ground cerebral tissues have been depressed by addition of 100 to 750 μM-chlorpromazine (BERNSOHN, NAMAJUSKA and BOSHES, 1956; CENTURY and HORWITT, 1956) and oxidative phosphorylation by 200 μM-chlorpromazine (BERGER, STRECKER and WAELSCH, 1956). In cell-containing cerebral tissues, labelling of phospholipids has proved more sensitive in being accelerated by 100 μM chlorpromazine (MAGEE, BERRY and ROSSITER, 1956). These concentrations are much higher than those of 5 to 10 μM at which chlorpromazine has now been shown to act on the electrically stimulated tissue. In all these situations, as *in vivo*, chlorpromazine is presumably concentrated in the tissue or tissue elements on which it is acting; data quoted in the Results section showed that concentrations of the drug reached *in vivo* and during the present experiments *in vitro*,

VOL
1
1956/

were comparable. A second phenothiazine, mepazine, was closely similar in action to chlorpromazine in the present systems.

Yohimbine (280 μM) has recently been found to affect ion movement in frog sciatic nerve during stimulation but not in its absence (SIMON, 1955). Cerebral tissues in the present experiments were again much more sensitive during stimulation. Active concentrations were much lower than in the peripheral nerve, for in cerebral tissues the selective depression of stimulated metabolism was obtained with 8μM yohimbine. This possibly indicates why, after systemic administration, yohimbine has central rather than peripheral effects; under other circumstances, it can act as a local anaesthetic and effects now seen in cerebral tissues show some resemblance to findings with cocaine (BOLLARD and MCILWAIN, unpublished).

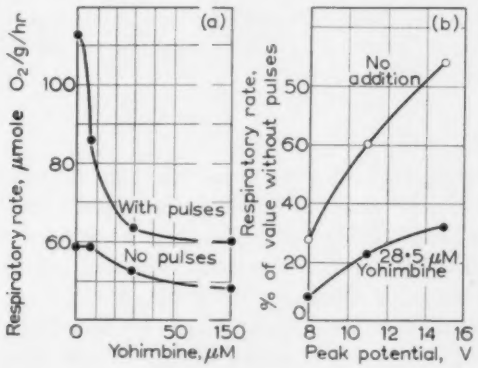


FIG. 3.—Yohimbine and the respiration and respiratory response of guinea pig cerebral tissues to alternating condenser pulses at 100/sec., of 0·4 msec. time-constant in concentric-electrode vessels E of AYRES and MCILWAIN (1953). a. Varying concentrations of yohimbine with and without pulses at a peak potential of 15 v. b. Voltage-response curves in presence and absence of yohimbine.

By contrast, there must be noted instances in which substances of marked central effects were without action in the isolated, electrically stimulated, cerebral tissue. Reserpine and pentamethylenetetrazole are of this category; also, methylethylglutarimide failed to antagonize the effects of low concentrations of butobarbitone. It thus appears that the interactions of reserpine with serotonin and adrenaline (PLETSCHER, SHORE and BRODIE, 1956; HOLZBAUER and VOGT, 1956) are not reflected in the present measures of metabolic response. This obtains even though relevant parts of the brain were examined; the release of bases, however, was not studied. It has been concluded on other grounds that electrical excitation as performed in the present experiments probably brings about a general depolarization of cerebral tissues and is less likely to display metabolic sequelae to transmission of nerve impulses (MCILWAIN, 1956*b*; BOLLARD and MCILWAIN, 1957). Thus substances whose action is not displayed in the present experiments may act preferentially on processes of transmission. Of actions which are exhibited, therefore, that of yohimbine may well be related to the effect of the drug on ionic movements related to polarization (SIMON, 1955).

Three further points may be noted about the specificity in metabolic action of the substances now examined. In the production of hydrallazine as a hypotensive agent

a hydrazine has been chosen which does not have the marked metabolic effects on cerebral tissues shown by other hydrazines. This may be relevant to its selection as a drug. Secondly, the introduction of a thio group, converting phenobarbitone to thiogardenal, is associated with loss of depressant action *in vivo*, and with loss of inhibition of metabolic response *in vitro* (compare McIlwain, 1953). No *in vitro* counterpart has however been found to the action of the thio compound as a convulsant. Thirdly, the depression of both respiration and glycolysis of specifically the electrically stimulated tissue by agents including butobarbitone, chlorpromazine and mepazine indicates that electrical pulses bring about a more profound change in the tissue's reactions than simply a sensitization to the drug. For with higher concentrations of butobarbitone, acting on tissue in the absence of pulses or other stimulating agents resulting changes are not those just described, but involve with the fall in respiration an increase in glycolysis. Thus several new *in vitro* counterparts to *in vivo* actions of drugs have been found, though they have not yet been found in all compounds in which they have been sought.

VOL
1
1956/

SUMMARY

(1) $\beta\beta$-Methylethylglutarimide did not antagonize the depression of respiration caused in cerebral tissues by butobarbitone. This applied to tissues stimulated electrically as well as to those without stimulation. Alteration in level of glycolysis caused by butobarbitone (in absence of electrical pulses, increased; or in their presence, decreased) was also unaffected by the imide.

(2) Electrically stimulated cerebral tissues were very sensitive to chlorpromazine and mepazine, respiratory response to pulses being inhibited by the compounds at 5 to 8 μM. These concentrations are only a small fraction of those which affect other metabolic properties of the tissues.

(3) Yohimbine also affected metabolic response to pulses when present in very low concentration, of some 8 μM. At 30 to 150 μM, respiration of unstimulated tissues was also slightly inhibited, a much greater action remaining against the stimulated tissue.

(4) Pentamethylenetetrazole, a convulsant barbiturate, reserpine and hydrallazine did not notably affect respiration or glycolysis.

Acknowledgements—The investigations were supported largely by the Research Fund of the Bethlem Royal Hospital and the Maudsley Hospital, for which we give grateful acknowledgment. Experimental assistance was given by Mr. A. McNiel and in part by Miss H. Martin.

REFERENCES

Anguiano G. and McIlwain H. (1951) *Brit. J. Pharmacol.* **6,** 448.
Ayres P. J. W. and McIlwain H. (1953) *Biochem. J.* **55,** 607.
Berger M., Strecker H. J. and Waelsch H. (1956) *Nature, Lond.* **177,** 1234.
Bernsohn J., Namajuska I. and Boshes B. (1956) *J. Neurochem.* **1,** 145.
Berti T. and Cima L. (1954) *Arch. int. Pharmacodyn.* **98,** 452.
Bollard B. and McIlwain H. (1957) *Biochem. J.* **66,** 651.
Century B. and Horwitt M. K. (1956) *Proc. Soc. exp. Biol.* **91,** 493.
Courvoisier S., Fournel F., Ducrot R., Kolsky M. and Koetschet P. (1953) *Arch. int. Pharmacodyn.* **92,** 305.
Dickens F. (1939) *Biochem. J.* **33,** 2017.
Dubost P. and Pascal S. (1953) *Ann. pharm. franç.* **11,** 615.

GANSHIRT H. and BRILMAYER H. (1954) *Arch. int. Pharmacodyn.* **98,** 467.
GEIGER A. and YAMASAKI S. (1956) *J. Neurochem.* **1,** 93.
GREENGARD O. and MCILWAIN H. (1955) *Biochem. J.* **61,** 61.
HOLZBAUER M. and VOGT M. (1956) *J. Neurochem.* **1,** 8.
MAGEE W. L., BERRY J. F. and ROSSITER R. J. (1956) *Biochim. biophys. Acta* **21,** 408.
MCILWAIN H. (1953) *Biochem. J.* **53,** 403.
MCILWAIN H. (1955) *Biochemistry and the Central Nervous System.* Churchill London.
MCILWAIN H. (1956*a*) *Physiol. Rev.* **36,** 355.
MCILWAIN H. (1956*b*) *Biochem. J.* **63,** 257.
NIESCHULZ O., POPENDIKER K. and SACK K. (1954) *Arzneimittel Forsch.* **4,** 232.
PLETSCHER A., SHORE P. A. and BRODIE B. B. (1956) *J. Pharmacol.* **116,** 46, 84.
SHAW F. H., SIMON S. E., CASS N., SHULMAN A., ANSTEE J. R. and NELSON E. R. (1954) *Nature, Lond.* **174,** 402.
SIMON S. E. (1955) *Aust. J. exp. Biol. med. Sci.* **33,** 79.
WOLLENBERGER A. (1955) *Biochem. J.* **61,** 77.

OL.
1
6/57

Journal of Neurochemistry, 1957, Vol. 1, pp 358-363, Pergamon Press Ltd., London.

THE ELECTROPHORETIC SEPARATION OF THE SOLUBLE PROTEINS OF THE BRAIN

D. M. ROBERTSON
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(*Received* 10 *March* 1957; *in revised form* 6 *June* 1957)

VOL
1
1956/

SEVERAL investigators have described the separation of the soluble proteins of the brain by paper electrophoresis, under different experimental conditions. KAPS (1954) separated six protein fractions but carried out no quantitative estimations; he reported an increased amount of 'albumin' in the brain tissue in the neighbourhood of tumours. DEMLING, KINZLMEYER and HENNING (1954) examined the proteins separated from rat brain tissue after partial autolysis. HOFMANN and SCHINKO (1956) investigated the soluble proteins of human and guinea pig brain. PALLADIN and

TABLE 1.—VALUES OBTAINED BY DIFFERENT INVESTIGATORS FOR THE DISTRIBUTION OF THE SOLUBLE PROTEINS OF THE BRAIN EXPRESSED AS A PERCENTAGE OF THE TOTAL SOLUBLE PROTEIN

Animal	Tissue	Fraction					Reference
		γ-Globulin	β-Globulin	α-Globulin	Albumin	Pre-albumin	
Rat	Cortex	9·4	27·8	47·5	15·3	—	DEMLING, KINZLMEYER and HENNING (1954)
Man	Cortex	18·9	66·5	12·0	1·9	0·7	HOFMANN and SCHINKO (1956)
Rat	Cortex and cerebellum	14·9	18·6	34·4	15·9	16·2	CARVAGLIOS and CHIAVERINI (1956)
Rat	Whole brain	6·7	34·5	31·6	19·0	8·2	Present investigation

POLJAKOVA (1956) reported differences between the soluble proteins of the brain and peripheral nerve. CARVAGLIOS and CHIAVERINI (1956) studied the soluble proteins extracted from rat brain tissue that had previously been freeze-dried. KIYOTA (1956) found a variation with age in the distribution of soluble proteins.

Most of these investigators have reported the presence of seven main protein fractions, but quantitative estimates of their relative amounts have varied widely. Values derived from the published data are given in Table 1. The different zones have generally been defined by reference to the proteins of serum, which are run simultaneously on the paper at the side of the brain proteins.

The purpose of the present investigation was to find the best conditions for the separation of the soluble proteins of the brain by paper electrophoresis, and to obtain further information about the relative quantities of the different types of proteins that

are present. The staining method of JENCKS, JETTON and DURRUM (1954) was used to estimate the protein in the separated zones. The values obtained by this method were confirmed by separate determinations of the nitrogen content.

METHODS

Protein extraction

(a) The whole brain from an adult Wistar strain albino rat was used in each zone electrophoresis experiment. After dipping in distilled water, the brain was dried by draining, weighed, cut into small pieces and frozen in solid carbon dioxide. The frozen tissue was ground to a fine powder and 5 ml of a buffer, containing 0·05 M-sodium barbitone, adjusted to pH 8·6 with citric acid, were added. After thawing, the mixture was centrifuged for 5 min at 3000 rev/min (6950 G) to remove the larger particles, and the supernatant was enclosed in a length of cellophane dialysis tube (VISKING, CRAMPTON & Co., Manchester) about 10 cm long and 2 cm diameter which was tied at the ends. The solution was concentrated to 2·5 ml by dialysis against a 50 per cent w/v solution of gum acacia in the buffer used for electrophoresis. This rapid method of concentration of protein solutions was previously used by FERUGLIO and RIMINI (1955). The concentrated solution was frozen and thawed three times in a centrifuge tube and then centrifuged at 3000 rev/min for 30 min. The sediment removed, which was insoluble in acetone, chloroform and 2 N-NaOH, contained by the micro-Kjeldahl method less than 10 per cent of the total nitrogen content of the extract. Kjeldahl estimations on extracts prepared by this method showed that the protein content corresponded to about 5 per cent of the total wet weight of the rat brain. This represents an extraction of about 50 per cent of the total protein in the tissue.

(b) The butanol extraction method of MORTON (1950) was applied by ADJUTANTIS (1954) to the preparation of lipid-poor, soluble protein extracts from rat liver. He reported that extracts prepared by this method gave a better resolution of the protein zones than was obtained with extracts prepared by other methods. In this method a single extraction of the tissue homogenate with excess *n*-butanol is followed by centrifugation, and dialysis to remove dissolved *n*-butanol. Morton believed the action of *n*-butanol was that of removing lipid, particularly of phospholipid, from the proteins, and bringing into solution proteins bound to tissue particles.

In the present experiments it was found possible to obtain six or seven separate protein zones by the electrophoresis of brain extracts prepared by Morton's method, but there was much loss of protein into the alcoholic phase during the extraction with excess *n*-butanol; three extractions with one-fifth of the volume of *n*-butanol were found to remove about 80% of the nitrogenous material of the extract. The previous method of preparing extracts was therefore preferred.

Electrophoresis

A zone electrophoresis apparatus was used in which strips of filter paper were held horizontally between the electrode compartments. Six strips, 9·5 cm × 47 cm, cut from a single sheet of Whatman No. 2 chromatography paper, were immersed in 0·05 M-sodium barbitone buffer, blotted, and arranged in pairs in three racks. Extract (0·07 ml) was applied on the edge of a 7 cm cover-glass to each paper, in a line about 3 cm from the negative end. The racks rested upon one another when in position in the apparatus, and the length of paper for all strips, from one electrode compartment to the other, was the same. This arrangement caused the same voltage gradient to be maintained along each paper strip, and each strip showed the same pattern of protein migration after staining.

Electrophoresis on paper inclined to the horizontal, or hung vertically, did not result in a reproducible protein pattern. The temperature of the apparatus was kept about 4° to restrict diffusion (BOYD, 1954). A potential gradient of 2·5 v/cm applied for 24 hr was found to give clearer separation of the main zones than a larger voltage applied for a short time, or a smaller voltage applied for a longer time.

Protein estimation

(a) *Biuret method.* The total protein in the extracts was estimated colorimetrically by the biuret method, using a volume of 0·05 ml. A standard curve was drawn, which showed the absorption, in the region of 5400 Å, of the biuret coloration given by amounts of the extract between 0 and 0·10 ml.

(b) *Dye method.* After zone electrophoresis the staining method of JENCKS, JETTON and DURRUM (1954) with bromophenol blue was used without modification. When the staining was complete the dyed strips were made translucent with medicinal paraffin and the light absorption found at intervals of one millimetre by means of a photoelectric absorptiometer. The protein content of the different zones could be found from the area under the absorption curve (Fig. 1), but quantitative protein values obtained by elution of the dye showed smaller standard deviation than those found by measuring the area under the absorption curve. For this purpose the paper was cut into strips at the points of least absorption on the curve. After the removal of paraffin with ethyl ether, the cut-out zones were eluted with 0·01 N-NaOH until all the dye was removed. The solutions were made up to 10 ml and the absorption values of the dye were determined with a photoelectric colorimeter at 6000 Å. Values for the protein in the zones were taken from a calibration curve.

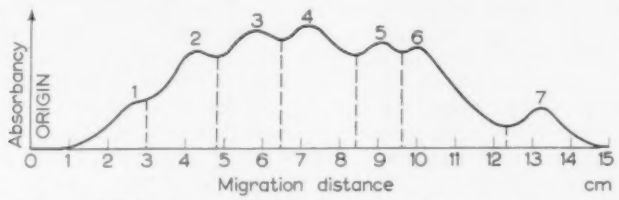


FIG. 1.—The colorimetric absorption curve of a representative zone electrophoresis strip, stained to show the migration pattern of the soluble proteins of rat whole brain.

(c) *Micro-Kjeldahl method.* Estimates of the protein distribution were confirmed by determining the nitrogen content of the protein zones. The electrophoresis strips were dried, stained and rinsed in the normal way, except that the final rinse in sodium acetate solution was omitted to prevent any re-conversion of residual diethyl-barbituric acid to the sodium salt, which is insoluble in the ethyl ether used to remove the remaining diethylbarbituric acid. The zones were cut out and numbered. The nitrogen content of each zone was found by the micro-Kjeldahl method, and a correction was made for the nitrogen contribution from the paper. It was checked that the dye had no nitrogen present as impurity. Some non-protein material in the vicinity of the 'pre-albumin' band and near the origin, which gave a positive ninhydrin reaction, was removed during the immersion in the aqueous dye bath.

RESULTS

Protein distribution and relative migration rate. Eight representative zone electrophoresis strips were selected, one from each of five experiments with extracts of rat whole brain. These were subjected to the staining procedure of JENCKS *et al.*, and the protein distribution in each strip was estimated by the dye elution method. For each zone, the distance migrated from the origin was expressed as a percentage of the distance migrated by human serum albumin under the same conditions. The zones were numbered 1–7, omitting the origin. The three remaining strips were used for the determination of the nitrogen content of the separated zones. Values for the protein distribution (expressed as percentage total protein) found by dye elution and nitrogen content, together with the standard deviations, are given in Table 2. The relative migration rates are also given.

In Fig. 2 the top photograph (A) shows an electrophoresis strip obtained with an extract of rat whole brain, prepared by grinding with sand at room temperature and centrifuging only. Concentration of the same extract by dialysis against gum acacia for one hour resulted in a change in the protein pattern (B). In (C) can be seen the further improvement in separation of protein zones produced by initial freezing of the tissue in solid carbon dioxide, and clarification of the extract by freezing and thawing three times after concentration by dialysis. In (D), (E), and (F) are shown the electrophoresis patterns obtained for the soluble proteins of rabbit, cat and sheep cortex,

VOL
1
1956/

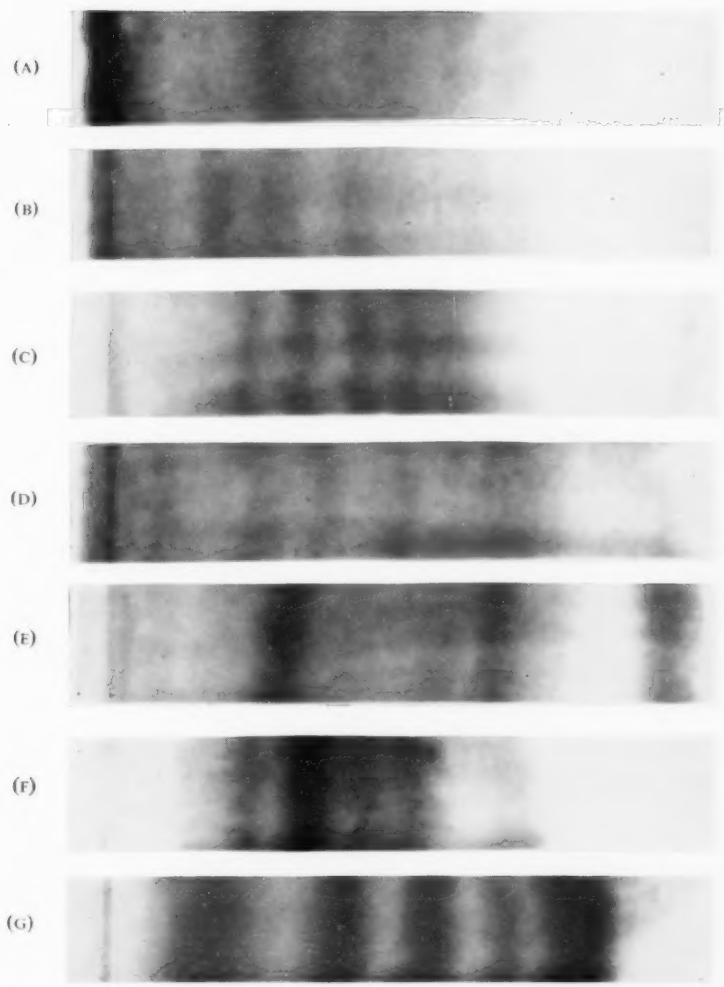


FIG. 2.—Some typical electrophoretic separations of the soluble proteins of the grey matter of rat, rabbit, cat, and sheep brain.

VOL
1
1956/

respectively, all prepared under the same experimental conditions as (C). The bottom pattern (G) is that of the proteins of human blood serum separated under similar conditions.

TABLE 2.—QUANTITATIVE PROTEIN DISTRIBUTION AND RELATIVE MIGRATION RATES OF SOLUBLE PROTEINS OF THE RAT BRAIN
(Mean values from five experiments)

Zone No. from origin	Analagous serum protein	Relative migration rate	S.D. for relative migration rate	Protein content of zone by dye method (%)	S.D. for protein content	Protein content by micro-Kjeldahl (%)	Protein (nitrogen) / Protein (dye)
1	γ-Globulin	15·4	1·4	6·7	0·8	6·0	0·90
2	β-Globulin	26·7	2·4	16·3	0·9	16·5	1·02
3	β-Globulin	38·6	2·3	18·2	0·8	19·5	1·07
4	α_2-Globulin	50·1	4·8	22·3	0·8	21·8	0·97
5	α_1-Globulin	60·1	3·8	9·3	0·7	8·4	0·90
6	Albumin	75·3	2·9	19·0	0·7	17·9	0·94
7	Pre-albumin	93·3	7·5	8·2	0·2	9·9	1·22

The relative migration rate is defined as the percent of the migration distance of serum albumin in the same time under the same conditions.

Freezing might be expected to cause the break up of labile lipoproteins: but freezing, thawing and centrifuging an extract of the soluble proteins of the rat brain, without preliminary concentration by dialysis against gum acacia in buffer solution, had no visible effect on the electrophoresis pattern. Protein extracts prepared from brain tissue frozen with solid carbon dioxide also resembled those from unfrozen brain in the intensity of staining of electropherograms stained for lipids with Sudan Black B in 50% ethanol. While this does not preclude the possibility that some change in the physical state of the lipoproteins may be brought about by freezing, it indicates that there is no serious loss of lipid from the lipoproteins as a result of freezing under the conditions used. It was also found by HOFMANN and SCHINKO (1956) that most of the lipoproteins remained in brain protein extracts after freezing to −78° in solid carbon dioxide.

ADJUTANTIS (1954) reported that the electrophoretic separation of liver proteins was improved by treatment with *n*-butanol. Extracts of rat brain proteins treated with *n*-butanol under the conditions used by ADJUTANTIS gave the same number of bands as extracts not treated in this way: but there was a significant change in the relative migration rates, and a higher proportion of protein was found in the band nearest the origin. The nature of the changes in the proteins produced by treatment with *n*-butanol is not known, but it clearly caused a considerable loss of lipoprotein from the extracts. The use of *n*-butanol therefore appeared to be unsatisfactory as a general method for the preparation of brain protein extracts for electrophoretic separation.

No quantitative difference was found in the protein distribution of fresh extracts of rat whole brain and extracts from brains kept at 4° for up to three days. There was, however, a steady decline in the sharpness of separation of the protein zones with the preserved tissue.

Staining conditions. Bromophenol blue showed no affinity for the paper, and gave a satisfactory elution of the dye from the protein. Electrophoresis strips dyed for protein with Amidoblack 10 B and naphthalene black, gave protein distribution values different from those obtained with bromophenol blue. The difference may be attributed to the partial loss of dye from the protein during the repeated rinsing that was necessary for the removal of the excess dye from the paper.

Buffer solutions. Of phosphate, borate, triethanolamine, glycine and barbitone buffers, the most effective in the electrophoretic separation of soluble brain proteins was a combination of sodium barbitone and citric acid. The use of acetic acid for the adjustment of buffer pH gave inferior separation.

Supporting media. Whatman chromatography paper No. 1, 2, 3 mm, 4, and 541 were tried in conjunction with the buffers mentioned above. Whatman No. 2 paper gave the most reproducible results for protein distribution; Whatman No. 1 gave a clearer definition of the zones near the origin but the 'pre-albumin' band was often difficult to see. Filter paper esterified with concentrated nitric acid, and partially acetylated papers were found to have no particular advantage over untreated paper. At the beginning of the present work it was intended that the method of BOYD (1954), which was developed for the study of serum lipoproteins, should be used. It was found, however, that the use of Whatman No. 3 paper in zone electrophoresis of the soluble proteins of the brain, with voltage gradients in the region of 8 v/cm for 8 hr separations gave poor resolution of the zones.

VOL
1
1956/

DISCUSSION

The main criteria used during the development of the method of zone electrophoresis described, were (a) good reproducibility of the migration pattern and (b) good separation of the soluble protein fractions of the brain. The maximum number of protein (i.e. dye-retaining) zones which were obtained on a zone electrophoresis strip in the course of the present work with rat whole brain was ten, but it was found difficult to repeat the separation of some of the less clearly defined minor zones. Electrophoretic conditions were therefore selected so as to favour the spacing-out of the seven major zones with a high degree of reproducibility. Under the conditions used the largest single fractions were those in zones 4 and 6 with relative migration rates of $50{\cdot}1 \pm 4{\cdot}8$ and $75{\cdot}3 \pm 2{\cdot}9$ respectively, which correspond in position to the 'α_2-globulin' and 'albumin' bands of human serum. Under the same conditions extracts of sheep, cat and rabbit brain also gave seven major zones, but the relative quantities of proteins in the different zones differed for each animal species. The relative migration rate of a protein fraction, defined as the ratio per cent of the observed migration distance to that of human serum albumin under standard conditions, was found to be sufficiently constant to serve as an index for the identification of the different protein fractions on an electrophoresis strip. This is analogous to the rate of flow (R_F) constant of a compound in paper chromatography.

Electropherograms prepared from the grey and white matter of sheep brain were closely similar in appearance. Each gave seven major bands in the same relative positions and only slight differences in the distribution of the proteins were observed. These observations agree with those of HOFMANN and SCHINKO (1956) who found only slight qualitative differences and no significant quantitative differences in the distribution of soluble proteins in extracts prepared from grey and white matter of human

and animal brains. CARVAGLIOS and CHIAVERINI (1956) found no difference between the soluble proteins of the cerebral cortex and cerebellum in the rat brain. The whole rat brain was therefore used in most of the present experimental work.

The different ratios for the quantitative distribution of proteins in the rat brain reported by different investigators arise mainly as a consequence of the different experimental conditions used. DEMLING, KINZLMEYER and HENNING (1954) used phosphate buffers in the course of their pioneering studies of the electrophoretic separation of soluble tissue proteins. CARVAGLIOS and CHIAVERINI (1956) extracted proteins from the cerebellum and cortex of rats using a process of freeze-drying, and they carried out an electrophoretic separation of the re-dissolved protein in borate buffer. In the present investigation it was found that alteration of any of the experimental conditions of electrophoresis, with the exception of temperature between the limits 4° to 18°, caused enough change in the protein pattern to give different quantitative results for the ratios of the zones. Alteration of the buffer strength or type, was the largest contributory factor to such quantitative variations. This indicates that the interaction that takes place between the soluble proteins of the brain and the constituents of the buffer solutions has a considerable influence on the electrophoretic properties of the proteins. It was shown by nitrogen determinations that the protein distribution indicated by the staining method with bromophenol blue approximates closely to the protein distribution based on nitrogen content.

In experiments on the electrophoretic separation of larger quantities of brain proteins using starch or agar as a supporting medium, it was found difficult to prevent the non-linear migration of protein zones, caused by the disturbance of buffer flow at the sides of the containing vessel. This effect is analogous to the faster flow of the central part of the zone in a chromatography column. Although milligram quantities of protein could be separated by this method, the proteins are contaminated by the medium used.

Acknowledgements—The author thanks Dr. D. RICHTER for his advice. He is indebted to Miss M. J. POLLEY for photography and the Medical Research Council for a personal grant.

SUMMARY

(1) The conditions affecting the electrophoretic separation of the soluble proteins of the rat brain have been investigated.

(2) The quantitative protein distribution was evaluated by a method of dye elution, and by estimating the nitrogen content.

REFERENCES

ADJUTANTIS G. (1954) *Nature, Lond.* **174,** 1054.
BOYD G. S. (1954) *Biochem. J.* **58,** 680.
CARVAGLIOS R. and CHIAVERINI P. (1956) *Experientia* **12,** 8.
DEMLING L., KINZLMEYER H. and HENNING N. (1954) *Z. exp. Med.* **122,** 416.
FERUGLIO F. S. and RIMINI R. (1955) *Bull. Soc. ital. biol. sper.* **31.**
HOFMANN G. and SCHINKO H. (1956) *Klin. Wschr.* **34,** 86.
JENCKS W. P., JETTON M. R. and DURRUM E. L. (1954) *Biochem. J.* **60,** 2.
KAPS G. (1954) *Arch. Psychiat. Neurol.* **192,** 115.
KIYOTA K. (1956) *Psychiat. et Neurol. jap.* **58,** 191.
MORTON R. K. (1950) *Nature, Lond.* **166,** 1092.
PALLADIN A. and POLJAKOVA N. (1956). *Dokl. Akad. Nauk. S.S.S.R.* **107,** 568.

Journal of Neurochemistry, 1957, Vol. 1, pp. 364-369 Pergamon Press Ltd., London

THE PERSISTENCE OF TAURINE IN THE BRAINS OF PYRIDOXINE-DEFICIENT RATS

D. B. HOPE*
Department of Pharmacology, Oxford University

(*Received* 2 *April* 1957)

VOL
1
1956/

THE amino acid taurine appears to have a very wide distribution in the animal kingdom, and recently it has also been discovered in certain algae (LINDBERG, 1955). The main interest, until recently, has centred round the occurrence of taurine in the liver, where it serves as a precursor of bile acids, e.g. taurocholic acid, but it is now known that many other tissues, vertebrate and invertebrate, contain free taurine.

The occurrence of taurine in nervous tissue has been studied in recent years. There are reports which show that this amino acid occurs in a high concentration in the axoplasm of the nerves of certain crustaceans and molluscs (LEWIS, 1952; KOECHLIN, 1955). In the mammalian brain, quantitative estimates are available for the cat (STEIN and MOORE, 1954) and the rat (AWAPARA, 1956); here the amounts found are between 50 and 100 mg per 100 g of fresh tissue.

Two enzymes are now known to be active in the decarboxylation of sulphur-containing amino acids. The first of these was described as L-cysteic acid decarboxylase of mammalian liver (BLASCHKO, 1942). This enzyme is now believed to be identical with the L-cysteine sulphinic acid decarboxylase of liver (CHATAGNER and BERGERET, 1951); the evidence for the identity of these two enzymes has already been presented (HOPE, 1955). Recent work by FROMAGEOT and his group has shown that a second decarboxylase which acts upon both L-cysteic and L-cysteine sulphinic acids is present in the brain (BERGERET, CHATAGNER and FROMAGEOT, 1955); this enzyme is probably identical with the L-glutamic acid decarboxylase present (DAVISON, 1956).

Some time ago, it was shown that the liver enzyme quickly disappears from rats kept on a diet deficient in vitamin B_6 (BLASCHKO, CARTER, O'BRIEN and SLOANE STANLEY, 1948); the disappearance of the enzyme is followed by the disappearance of taurine from the urine (BLASCHKO, DATTA and HARRIS, 1953).

In order to obtain information on the biological significance of the high concentration of taurine in nervous tissue, it was decided to investigate the concentration of the amino acid in the brains of normal and pyridoxine-deficient rats. The results of these experiments led to a re-investigation of the urinary taurine excretion in deficient animals; these experiments will also be reported.

The paper contains a description of the method adopted for taurine estimation; this is based on the work of HARRIS, MITTWOCH, ROBSON and WARREN (1955) and that of AWAPARA (1956).

* Present address: M.R.C. Radiobiological Research Unit, Atomic Energy Research Establishment, Harwell, Didcot, Berks.

MATERIALS AND METHODS

Animals

The rats used in these experiments were of the hooded Lister strain obtained freshly weaned from two sources: (i) The Department for Research in Industrial Medicine, M. R. C. Laboratories, Hampstead and (ii) The British Ratin Co. Ltd., East Grinstead, Sussex. The pyridoxine-deficient diet has already been described by BLASCHKO *et al.* (1953). The animals on arrival in the Laboratory were put on the pyridoxine-supplemented diet for 12 days; in this period they grew rapidly and they attained a weight of about 90 g. Some of the animals were reared on a diet of rat-cake; these will be referred to as normal animals. The pyridoxine requirements of these animals are assumed to have been fully satisfied.

The urine from each rat was collected daily as described elsewhere (HOPE, 1957). The animals were sacrificed by decapitation, and the brain was removed immediately; the olfactory lobes were not included.

Substances

Taurine was recrystallized three times from water for use as a standard.

Cystamine hydrochloride was a gift from Prof. Z. M. BACQ upon whose suggestion this substance was administered to pyridoxine-deficient rats in one series of experiments. Phosphorylethanolamine and glycerylphosphorylethanolamine were kindly given by Dr. S. P. DATTA and Dr. R. M. C. DAWSON respectively.

Determination of taurine

(a) *Preparation of tissue extracts and chromatography.* Tissue extracts were prepared by homogenizing 1 g of tissue with 10 ml of an aqueous solution of picric acid (1 per cent, w/v) (HAMILTON, 1945). The precipitated proteins were removed by centrifugation; the sediment was washed once with 5 ml of the picric acid solution. The excess picric acid in the combined supernatants was removed by passage through a column (0·9 cm diameter and 2 cm high) of Dowex 2 × 8 (200–400 mesh) in its chloride form (STEIN and MOORE, 1954); the column was washed with picric acid solution (5 ml).

The aqueous solutions thus obtained were concentrated to about 1 ml and passed through a column (0·9 cm diameter and 4 cm high) of Dowex 50 × 8 (200–400 mesh) in the acid form; the column was then washed with 6 ml of water; this procedure elutes the taurine quantitatively (AWAPARA, 1956). The eluates were evaporated to dryness on a water bath and the residue was taken up in 1·0 ml of water.

Taurine was determined in urine by passing measured portions through a column of Dowex 50 as described above. The final extracts derived from tissues contained other acidic ninhydrin-positive substances, including phosphorylethanolamine, glycerylphosphorylethanolamine and substances so far unidentified.

Taurine was separated from these other acidic amino acids by chromatography on Whatman No. 4 filter paper with 'Analar' grade phenol saturated with water as the solvent system. The chromatographic procedure adopted was similar to that described by DENT (1948). The cabinets were designed to hold six large sheets (57 × 46·5 cm) of paper at the same time. Chromatography was carried out in a constant temperature room at 24 ± 1°C.

When brain extracts were used, aliquots of either 25 or 50 μl were applied to the paper; when urine was used the volume was 100 μl. These fluids were applied in the form of spots 1·5 cm in diameter. Three or four standards, 10–50 μl of a 0·1 per cent solution of taurine, were also applied on each chromatogram. The spots were placed 5 cm apart. In every sheet, one area was left blank, in order to provide a control for the colorimetric estimation.

The chromatograms were run overnight, with 33 ml of the solvent system for each sheet. The chromatograms were then thoroughly dried in a drying frame suspended over an electrically heated element (500 w) of nichrome wire loosely wound on an asbestos mill-board.

(b) *Development of the taurine spots.* The dry chromatograms were dipped into the following solution: 0·4 g ninhydrin, 95 ml acetone, 5 ml glacial acetic acid and 5 ml water, as described by HARRIS *et al.* (1955); the papers were allowed to dry in air for 30 min. The ninhydrin colour was developed by heating the chromatograms for 30 min on the surface of a 10 l. aspirator bottle full of water at 70°C, as described by BRONK (1955). The chromatograms were then dipped into a reagent

containing 2 ml saturated aqueous copper nitrate, 0·2 ml nitric acid (10 per cent v/v) and 100 ml acetone. After drying in air, the spots produced by taurine and the corresponding region of the blank were cut out in strips of equal size. Each strip was extracted by immersion in 5 ml of methyl alcohol for 1 hr with occasional shaking. The optical densities of the extracts were measured at 510 mμ. The optical density was directly proportional to the amount of taurine, up to 50 μg. Minor variations in the intensity of the colours were obtained from day to day; these variations were accounted for by construction of a calibration curve for each chromatogram.

RESULTS

Taurine in normal rat brain. In all experiments, the taurine concentration was estimated in the total amount of brain tissue obtained from each animal. The results are given in Table 1; it can be seen that the taurine concentration varies somewhat

TABLE 1.—TAURINE CONTENT OF NORMAL RAT BRAIN
Hooded Lister strain

Sex	Body wt. (g)	Brain wt. (g)	Taurine conc. (mg per 100 g wet tissue)
F	225	1·52	67·5
F	192	1·62	53·5
M	255	1·67	81·0
M	297	1·72	54·3

from rat to rat. The average concentration, 64·1 ± 13·1 mg per 100 g of wet tissue, is in good agreement with the figure of 68·7 ± 3·7 mg per 100 g of wet tissue recently reported by AWAPARA (1956).

Taurine in pyridoxine-deficient rat brain. The rats used in these experiments had been fed on the pyridoxine-deficient diet for 11 weeks. The animals had stopped

TABLE 2.—TAURINE CONTENT OF BRAIN FROM PYRIDOXINE-DEFICIENT RATS
Hooded Lister strain

Sex	Body wt. (g)	Brain wt. (g)	Taurine conc. (mg per 100 g wet tissue)
F	60	1·35	71·0
F	99	1·50	57·4
F	97	1·45	70·7
M	105	1·55	65·0
M	85	1·50	60·4

growing after four to five weeks; the weight then slowly declined; all the animals had acrodynia and the fur showed signs of pyridoxine deficiency. The results of the taurine determinations are shown in Table 2. The average concentration of taurine in pyridoxine-deficient rat brain was 64·9 ± 6·0 mg per 100 g of wet tissue. This shows that on the pyridoxine-deficient diet no depletion in taurine had occurred in this tissue.

Urinary excretion of taurine by rats in pyridoxine deficiency. The observation that

VOL 1 1956/

in severely pyridoxine-deficient animals the brain showed a normal taurine content was unexpected. It was therefore decided to re-investigate the urinary excretion of taurine with the more sensitive method described above.

The urines from rats deficient in pyridoxine for 14 weeks were collected and the daily excretion of taurine was determined.

The results of six determinations are shown in Table 3. It was found that in the

TABLE 3.—URINARY TAURINE EXCRETION BY PYRIDOXINE-DEFICIENT RATS
Hooded Lister strain

Sex	Body wt. (g)	Volume of urine in 24 hr (ml)	Taurine excreted in 24 hr (mg)
F	115	3·0	0·7
F	100	3·0	0·3
F	117	5·6	1·4
F	82	5·2	1·2
F	84	10·5	2·1
M	90	10·5	1·0

urines of all the animals examined, taurine continued to be excreted in small amounts. The average daily excretion of taurine was 1·1 mg per rat. In a control series five animals on the experimental diet, supplemented by daily additions of pyridoxine, excreted a mean of 18·6 mg daily; this figure is somewhat higher than that of 7·75 mg recently reported by AWAPARA (1956).

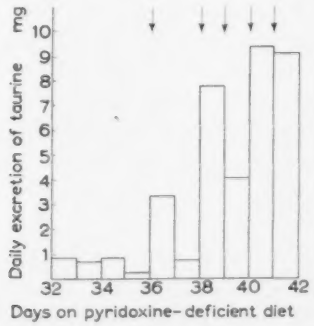


FIG. 1.—Urinary excretion of taurine in pyridoxine deficiency before and after the injection of cystamine (10 mg per day); each arrow denotes one administration of cystamine. The diagram shows the total amounts of taurine excreted daily.

The metabolism of cystamine in pyridoxine-deficient rats. It is known that cystamine, the terminal component of coenzyme A, can be converted to both hypotaurine (β-aminoethane sulphinic acid) and taurine in the normal rat (CAVALLINI, MONDOVI and DEMARCO, 1954; ELDJARN, 1954). Upon the suggestion of Prof. Z. M. BACQ, determinations were carried out of the urinary excretion of taurine in pyridoxine-deficient rats after the administration of cystamine. This was done in order to find out whether pathways of taurine formation were possible which were independent of the L-cysteic acid decarboxylase.

Cystamine was injected, as its hydrochloride, in two rats; 10 mg of the free base in 0·5 ml was administered in each subcutaneous injection. The urine was collected every 24 hr. Fig. 1 gives the results of taurine determinations in one animal, deficient in pyridoxine for 32 days, in which five single injections of cystamine were given.

It can be seen that the administration of cystamine led to a considerable increase in the amount of taurine excreted in the urine. If a complete conversion of the cystamine to taurine had taken place, 82 mg of taurine would have been expected; the amount of taurine recovered was 30·7 mg or, in other words, 37·5 per cent of the total cystamine administered could be accounted for as taurine excreted.

DISCUSSION

The striking result of the experiments reported is that the taurine concentration in the brain of the pyridoxine-deficient animals remained unchanged. The brain, therefore, is able to maintain its taurine level when the liver enzyme responsible for taurine formation had entirely disappeared. There can be little doubt that this observation finds its explanation in the discovery by FROMAGEOT and his colleagues, already mentioned in the introductory chapter, of a brain enzyme capable of decarboxylating cysteic and cysteine sulphinic acids. This enzyme, in contrast to the liver enzyme, is known to persist in pyridoxine-deficiency; this was first shown for the L-glutamic acid decarboxylase by ROBERTS, YOUNGER and FRANKEL (1951), and has since been confirmed by BERGERET *et al.* (1955), although with L-cysteine sulphinic acid as substrate the enzymic activity could only be demonstrated in the presence of added pyridoxal phosphate.

If we assume with DAVISON (1956) that the cysteine sulphinic enzyme is identical with the glutamic enzyme we must accept the remarkable fact that, in the brain, the enzyme is responsible for the formation of two entirely different products which are normally found in nervous tissue, namely, γ-aminobutyric acid and taurine. However, this interpretation cannot be considered as fully established until the enzyme has been isolated. It is of interest here, to mention that SÖRBO and HEYMAN (1957) have recently discussed the possibility that the liver enzyme responsible for the decarboxylation of L-cysteic acid is distinct from that which acts upon L-cysteine sulphinic acid.

The functional significance of the high concentration of taurine in nervous tissue is not known. The fact that glutathione seems to be rapidly oxidized in nervous tissue may mean that the breakdown of its cysteine moiety provides the source of the taurine. No facts are known at present which suggest that a specific function can be assigned to taurine.

That taurine is an end product of metabolism is confirmed in the present work. The earlier observation that in pyridoxine deficiency the urinary excretion of taurine falls was confirmed, but it has now been shown that it does not entirely cease in pyridoxine-deficient animals; the excretion continues at a reduced level. That the excretion was not noted by BLASCHKO *et al.* (1953) may well be due to the fact that the ninhydrin reagent is relatively ineffective in detecting small amounts of taurine (KAY, HARRIS and ENTENMAN, 1956). Since the liver enzyme disappears in the early stages of deficiency, the taurine present in the urine in the later stages is probably dependent upon the activity of the decarboxylase present in the nervous tissues, which persists under these conditions.

VOL
1
1956/

Taurine may also be derived from the cysteamine moiety of coenzyme A. The work of ELDJARN (1954) and CAVALLINI *et al.* (1954) has already shown that cystamine can be converted into taurine; in the present work, it has been found that in the pyridoxine-deficient animal a considerable proportion of cystamine finds its way into the urinary taurine. The possibility, therefore, that a second pathway of taurine formation exists must be considered; this has also recently been postulated, on other evidence, by CHATAGNER and BERGERET (1956).

SUMMARY

(1) The concentration of taurine in the brains of normal and pyridoxine-deficient rats has been determined. It was found that the taurine level does not change in an advanced stage of pyridoxine deficiency.

(2) The observation that the urinary excretion of taurine decreases in pyridoxine-deficiency, was confirmed. However, it was found that small amounts of taurine continued to be excreted; it is suggested that a continued formation of taurine in the brain contributes to this excretion at a lower level.

(3) Evidence is given that pyridoxine-deficient rats are able to form relatively large amounts of taurine from cystamine.

Acknowledgements—I wish to thank Prof. J. H. BURN, F.R.S., for the privilege of working in his laboratory and Dr. H. BLASCHKO for his advice and interest in this work. I am grateful to Dr. J. AWAPARA for making available information about the determination of taurine then unpublished. The work was carried out during the tenure of a Medical Council scholarship.

REFERENCES

AWAPARA J. (1956) *J. biol. Chem.* **218,** 571.
BERGERET B., CHATAGNER F. and FROMAGEOT C. (1955) *Biochim. biophys. Acta* **17,** 128.
BERGERET B., CHATAGNER F. and FROMAGEOT C. (1956) *Biochim. biophys. Acta* **22,** 329.
BLASCHKO H. (1942) *Biochem. J.* **36,** 571.
BLASCHKO H., CARTER W. C., O'BRIEN J. R. P. and SLOANE STANLEY G. H. (1948) *J. Physiol.* **107,** 18-19P.
BLASCHKO H., DATTA S. P. and HARRIS H. (1953) *Brit. J. Nutr.* **7,** 364.
BRONK J. R. (1955) D. Phil. Thesis, Oxford.
CAVALLINI D., MONDOVI B. and DEMARCO C. (1954) *Ric. sci.* **24,** 2649.
CHATAGNER F. and BERGERET B. (1951) *C. R. Acad. Sci., Paris* **232,** 448.
CHATAGNER F. and BERGERET B. (1956) *Bull. Soc. chim. biol., Paris* **38,** 1159.
DAVISON A. N. (1956) *Biochim. biophys. Acta* **19,** 66.
DENT C. E. (1948) *Biochem. J.* **43,** 169.
ELDJARN L. (1954) *Scand. J. Clin. Lab. Invest.* **6,** Suppl. No. 13.
HAMILTON P. B. (1945) *J. biol. Chem.* **158,** 375.
HARRIS H., MITTWOCH U., ROBSON E. B. and WARREN F. L. (1955) *Ann. hum. Genet.* **19,** 196.
HOPE D. B. (1955) *Biochem. J.* **59,** 497.
HOPE D. B. (1957) *Biochem. J.* **66,** 486.
KAY R. E., HARRIS D. C. and ENTENMAN C. (1956) *Arch. Biochem. Biophys.* **63,** 14.
KOECHLIN B. A. (1955) *J. biochem. biophys. Cytology* **1,** 511.
LEWIS P. R. (1952) *Biochem. J.* **52,** 330.
LINDBERG B. (1955) *Acta. Chem. Scand.* **9,** 1323.
ROBERTS E., YOUNGER F., and FRANKEL S. (1951) *J. biol. Chem.* **191,** 277.
SÖRBO B. and HEYMAN T. (1957) *Biochim. biophys. Acta* **23,** 624.
STEIN W. H. and MOORE S. (1954) *J. biol. Chem.* **211,** 915.

Journal of Neurochemistry, 1957, Vol. 1, pp. 370-376, Pergamon Press Ltd., London

HISTOCHEMICAL STUDY OF CHANGES OCCURRING DURING THE DEGENERATION OF MYELIN*

M. WOLMAN
Department of Pathology of the Hebrew University, Hadassah Medical School, Jerusalem, Israel

(*Received* 13 *April* 1957)

VOL.
1
1956/5

ON THE basis of histochemical and biochemical studies (WOLMAN, 1956*a* and *b*) it has been suggested that the normal myelin sheath contains an acidic polysaccharide, and that this compound is liberated during demyelinization. In order to test this, the effect of treatment of tissues by various osmium tetroxide reagents was studied (WOLMAN, 1957) and a method for the differential staining of acidic tissue compounds was worked out (WOLMAN, 1956*c*).

This paper deals with the application of these new techniques to the study of the changes occurring during the degeneration of myelin in the rat.

MATERIAL AND METHODS

The material and the technique were similar to those described previously (WOLMAN, 1956*b*). The right sciatic nerves and the right cerebral frontal lobes of rats were cut across, and after varying intervals of time (between 1 and 30 days) the animals were killed and the transected tissues were studied in comparison with the contralateral, presumably normal ones. Groups of three white rats of local breed were operated on the same morning and sacrificed together. Altogether 27 rats were used.

Pieces of tissue were put (a) into 96% ethanol for 4 days followed by immersion in absolute ethanol and xylene (two changes, about 30 min each) and embedding in paraffin (three changes at 58–60°, about 2½ hr altogether): (b) into 10% formalin (a 1 : 10 dilution of 36–39% formaldehyde in tap water) for 2 days, then rinsed for 2 days in running tap water, followed by dehydration (24 hr in 80% ethanol, 6 hr in 96% ethanol and 12 hr in 100% ethanol) and embedding in paraffin as above.

Sections were cut at 8 μ and stained by the following methods: (1) the Bi-Col method (WOLMAN, 1956*c*); (2) 1% solution of osmium tetroxide in carbon tetrachloride for 24 hr (WOLMAN, 1957); (3) for 7 days in SWANK and DAVENPORT's (1934) solution; (4) Sudan black B in 70% ethanol; (5) OsO_4 vapour, by leaving deparaffinized dry sections for 24 hr in a staining dish (80 ml) which contained at the bottom about 5 ml of a 1% aqueous osmium tetroxide solution; (6) leaving the dry sections for 24 hr in a staining dish which contained 5 ml of SWANK and DAVENPORT's solution; (7) haematoxylin and eosine, for orientation.

In each of the methods used, all the sections were stained simultaneously, so as to ensure uniform conditions of staining.

RESULTS

(1) *Bi-Col staining.* In the sections of normal, not transected nerve and brain (control side) *fixed in formalin*, both in the nerve and in the brain, the myelin sheath did not take up the brown stain. There was some faint yellowish or bluish tinge in some areas, and no staining in others. In Bi-Col stained sections of the *transected* nerve, no brown staining of the myelin sheaths was seen during the period of observation. In the brain, degenerating myelin sheaths appeared orange in the sections of

*This study was supported by research grants from the Hadassah Medical Organization, Jerusalem, and from the National Multiple Sclerosis Society, New York Grant No 138 R.

OL.
1
6/57

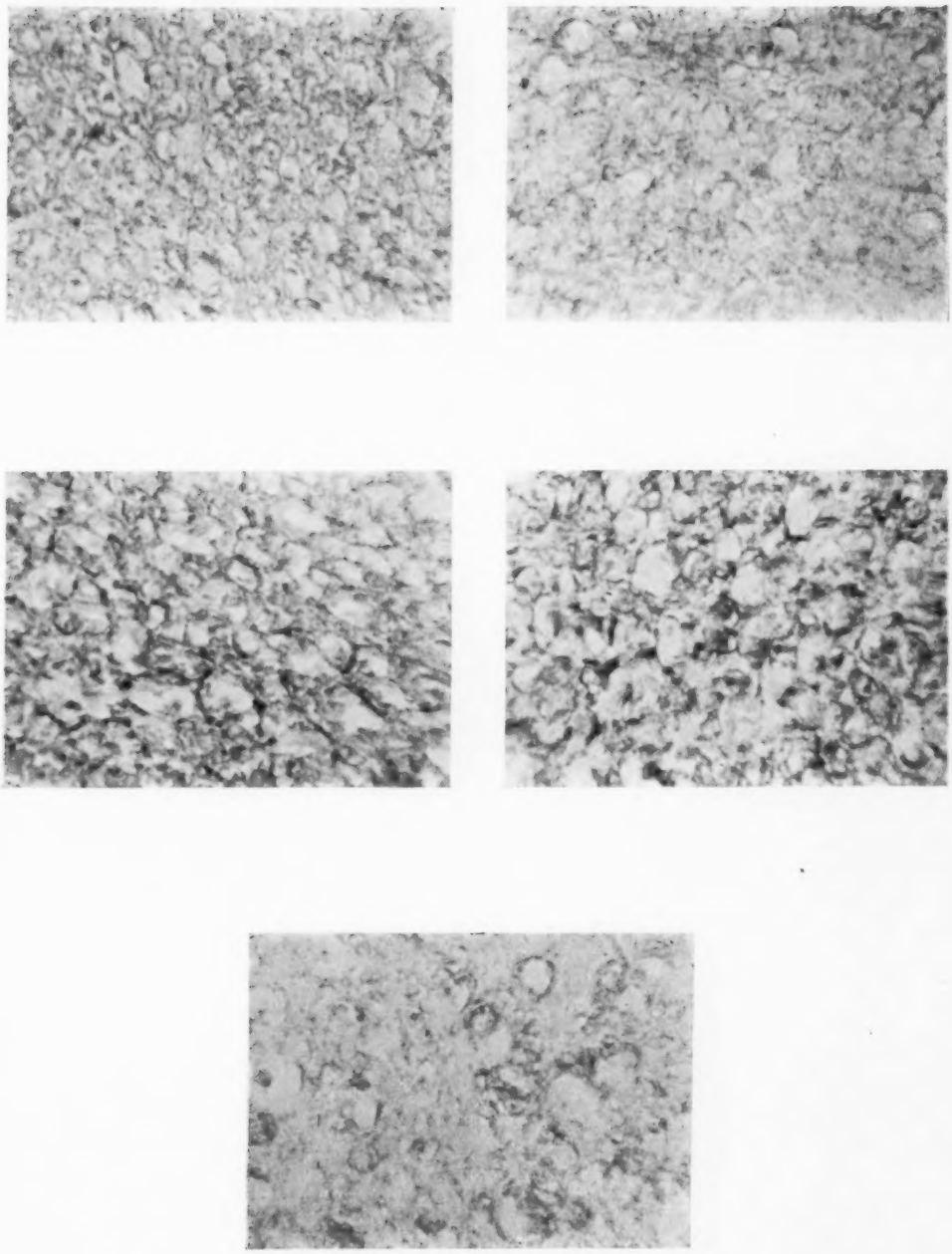


FIG 1.—Sections of rat sciatic nerves stained with Bi-Col. Conditions of staining, photography and development were identical for all sections. Top left—normal, not-transected nerve. Top right—nerve 1 day after transection. Middle left—2 days after transection. Middle right—4 days after transection. Bottom—6 days after transection.

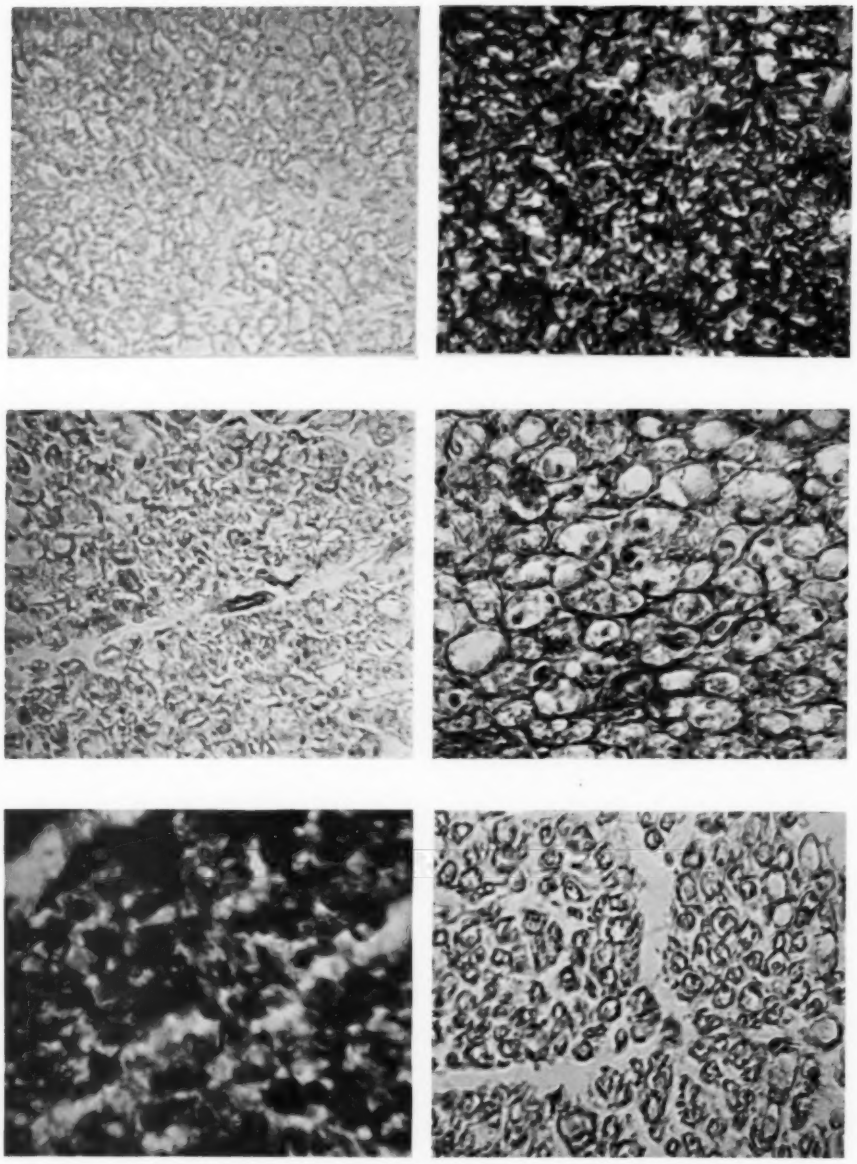


FIG. 2.—Sections of rat sciatic nerves stained with 1 per cent OsO_4 in CCl_4. Conditions of staining, photography, development and printing were identical for all sections. Top left—normal, not-transected nerve. Top right—nerve 1 day after transection. Middle left—4 days after transection. Middle right—6 days after transection. Bottom left—16 days, and bottom right—20 days after transection.

VOL.
1
1956/5

the animals killed 1 day after operation. The intensity of the staining increased in the subsequent days and the myelin sheaths appeared granular and studded with dark red-brown granules. This staining reached its maximal intensity on the fourth day and decreased subsequently, disappearing completely between the twelfth and sixteenth day. As from the fourth day brown-red granules could also be seen in microglial cells.

In the control sections of material *fixed in ethanol* the myelin sheaths stained light-brown both in the nerves and in the brain. In sections of the operated side, the degenerating myelin stained darker brown from the first day after operation. The appearance of the sheaths was again granular. The staining of degenerating myelin was most intense about the fourth day, and decreased markedly after the sixth day. By the sixteenth day the degenerating myelin sheaths did not stain at all or stained very lightly (Plate 1).

(2) *Staining with osmium tetroxide in carbon tetrachloride.* In the *formalin fixed* material from nerve and brain the degenerating myelin seemed to stain slightly more intensely than normal myelin between the second and sixteenth day. In the *alcohol fixed* material the degenerating myelin stained more heavily (brown to dark brown) than normal myelin between the first and sixteenth days. As can be seen in Plate 2, the staining was intense on the first day, but seemed to be localized mainly in the ground substance, between the myelin sheaths. This staining decreased rapidly and could hardly be seen in the 'two days' section. Dark staining of the myelin was first visible on the sixth day, was maximal about the twelfth day and decreased thereafter. On the thirtieth day the intensity of staining was so light that it could not be demonstrated when photographed.

(3) *Staining with* SWANK *and* DAVENPORT's *solution.* No staining was obtained in either the formalin or the alcohol fixed tissues.

(4) *Staining with Sudan black B.* In the *formalin fixed* sections the normal myelin sheaths stained brown. In the degenerating myelin the sudanophilic material appeared granular and progressively lighter with the passage of time from the operation. By the twelfth day most of the degenerating sheaths did not stain at all, while dark staining granules were sparsely spread along the empty envelopes.

In the *ethanol fixed* sections myelin sheaths stained pale yellow and were almost invisible.

(5) *Staining with vapour of osmium tetroxide.* In both the formalin and ethanol fixed tissues the myelin sheaths stained pale yellow. There was no appreciable difference between the degenerating and the normal myelin until the twentieth day. From that period the degenerating myelin sheaths failed to stain.

(6) *Staining with vapour of* SWANK *and* DAVENPORT's *solution.* The results differed in various experiments, probably because of different concentrations of the OsO_4 and of the ClO_2, depending on the amount of the solution at the bottom, its freshness, the tightness of closure of the staining jar, and the temperature.

In *formalin fixed* tissues, with high concentration of OsO_4 vapours, normal myelin of nerve stained brown, axons dark brown, and the brain tissue stained almost uniformly black. With low concentrations of osmium tetroxide vapour, myelin stained yellow to pale brown both in the nerve and cerebrum. Degenerating nerve-myelin stained similarly, while in the brain dark brown granules were found in the degenerating myelin and in gitter-cells between the eighth and the twentieth day.

In the *ethanol fixed* tissues moderate differences in concentration of osmium tetroxide vapours did not markedly affect the results. Normal myelin stained pale yellow; degenerating myelin stained darker than normal myelin (brown to black). The changes in the intensity of staining of degenerating myelin followed a similar pattern observed in the staining by osmium tetroxide in carbon tetrachloride.

COMMENT

Histochemical significance of the staining procedures. The Bi-Col staining method (WOLMAN, 1956*c*) permits differentiation between acidic groups of different dissociation constants. Strongly acidic groups, such as free sulphate (for example in chondroitin sulphate) and phosphate (such as in nucleoproteins) are stained blue; weakly acidic groups (for example in hyaluronic acid) are stained red to dark brown. Staining with Sudan black B is one of the best methods available for lipids.

TABLE 1.—DIFFERENTIATION OF GLUCOSE DERIVATIVES BY DIFFERENT OSMIUM TETROXIDE TREATMENTS

Compound	OsO_4		SWANK and DAVENPORT's solution		OsO_4 in CCl_4
	Aqueous solution	Vapour	Aqueous solution	Vapour	
Glucose	0	0	0	0	0
Glucosamine	0	0	0	+	+
Glucosaminic acid	+	+	±	+	±

+ means black or brown staining; ± means weak staining.
0 means no staining.

The interpretation of staining with osmium tetroxide and osmium tetroxide-oxidant mixtures (SWANK and DAVENPORT's solution) has been discussed elsewhere (WOLMAN, 1957). For the purpose of the present discussion the following points are pertinent.

On the assumption that a polysaccharide is split off during demyelinization and is responsible for the Marchi staining, a differentiation between some such compounds is possible by the use of osmium tetroxide in various forms. Table 1 (compiled from the results of previous study, WOLMAN, 1957) shows that glucose is not stained by any of the procedures. Glucosamine is not stained by either the vapour or solution of OsO_4; it is not stained by SWANK and DAVENPORT's solution, but is stained by the vapours of that solution (i.e. by a mixture of OsO_4 and ClO_2 gases). Glucosaminic acid is stained by all the forms of OsO_4 tested. The effect of OsO_4 in carbon tetrachloride is similar to that of the vapours of SWANK and DAVENPORT's solution, probably because of the presence of oxidizing impurities in the solvent.

It should be recalled that the difference between the effect of the osmium treatments in aqueous and non-aqueous media occurs also in tissue sections exposed to aqueous osmium solutions (WOLMAN, 1957): non-hydrated insoluble compounds are exposed in these solutions to the action of osmium in a non-aqueous medium and behave accordingly.

It is reasonable to assume therefore that insoluble compounds containing hexosaminic acids (and possibly other hexosamine derivatives) are stained both by OsO_4 and by SWANK and DAVENPORT's mixture, while hexosamine-containing compounds are stained by SWANK and DAVENPORT's mixture and not by OsO_4.

Findings during the early phase of degeneration of myelin. As from the first day of degeneration the myelin sheath in the rat seems to contain an increased amount of a weakly acidic compound which stains red-brown with the Bi-Col procedure. This compound, which is present in normal myelin, is probably liberated from the myelin complex during degeneration and its amount in free form increases until about the fourth day and decreases subsequently until its complete disappearance about the sixteenth day. During the first 6–8 days of degeneration, when the concentration of this compound is greatest, the osmium staining methods do not show any difference between normal and degenerating myelin, although a clear-cut difference is seen between the appearance of the ground substances of the normal and degenerating nerve tissue on the first day after transection.

VOL. 1 56/57

It is obvious, therefore, that the compound either does not stain with the osmium reagents used, or else it stains with osmium like normal myelin. Furthermore, the compound is not a lipid (although it may contain lipid moieties), because it does not stain with Sudan black, and also because it is better demonstrated in alcohol fixed tissues than in the tissues which went through the aqueous formalin procedure. The compound is apparently water-soluble as it could not be found in the nerves which were kept for 4 days in aqueous media (2 days formalin + 2 days in running water) and in the brain it disappeared from some places and remained in more central areas, and in areas where its concentration seemed to be high. The staining with gold of degenerating myelin in the brain of formalin fixed and thoroughly washed tissue, and the absence of similar staining of normal myelin, may also be explained by the assumption that aqueous medium does not completely remove the higher concentration of the compound in the degenerating myelin. It is possible, though, that the effect of formalin fixation is not due to the aqueous medium but rather to chemical reaction occurring between the compound or its parent complex and the formalin solution. Such reactions involving solubilization of polysaccharides, or their splitting from protein complexes by formaldehyde have been reported (UZMAN, 1955; FEYRTER, 1955).

It may be concluded that one of the earliest changes occurring in degenerating myelin in the rat is a liberation of a compound which is insoluble in lipid solvents, probably moderately soluble in water, and which contains weakly acidic groups. The compound is probably a polyuronic polysaccharide. Its amount diminishes from the fourth day after transection.

Findings during the second phase (the Marchi phase) of myelin degeneration. As from the sixth to the eighth day of degeneration the myelin sheath of the rat nerve contains compounds which stain by the SWANK-DAVENPORT mixture and by OsO_4 in CCl_4. In the present experiments staining by an aqueous SWANK-DAVENPORT solution was not successful, probably because of diffusion of the stainable material into the staining solution. This is apparently the reason why in the Marchi methods the staining has to be performed on blocks rather than on sections. As has been shown here, Marchi staining can be performed on slides providing the staining is performed in a non-aqueous medium. The solubility in water of the compound

responsible for the Marchi staining (except when contained in gitter-cells) is well known (SMITH *et al.*, 1956). Besides, the fact that degenerating myelin did not stain more intensely than normal myelin in the formalin fixed nerve (while the difference was observed in formalin fixed brain) also indicates that the compound responsible for staining is most probably moderately water-soluble. Staining with vapours of SWANK and DAVENPORT's solution, as well as staining with OsO_4 dissolved in carbon tetrachloride revealed that the Marchi stain made its first appearance in rat tissue about the eighth day and disappeared about the twentieth day.

Here again, the lack of staining of the 'Marchi compound' with Sudan black as well as its solubility characteristics (i.e. its solubility in water and its lack of solubility in lipid solvents) indicate that the compound is not a lipid (although lipid moieties might be contained in it).

DISCUSSION

The process of myelin degeneration after transection of nerves in the rat proceeds in the following stages:

Stage 1 lasts for about 8 days. During this stage there is a rapid loss of the orderly 'crystalline' structure of myelin in the rat with the liberation of a weakly acidic compound. Results of previous experiments (staining with P.A.S., WOLMAN, 1956*b*) combined with those presently reported, indicate that the compound is a polyuronic carbohydrate (possibly in combination with protein or lipid). This compound is insoluble in lipid solvents and is moderately soluble in water. The light staining of the compound with osmium tetroxide and the fact that defatted myelin stains similarly, speak in favour of the presence of amino groups in it. The amount of this polyuronic carbohydrate (or polyuronic amino-sugar) in the degenerating myelin reaches a peak about the fourth day after transection, decreasing thereafter.

Stage 2 lasts for about 12 days. During this stage the Marchi compound makes its appearance. This compound also has the characteristics of a polysaccharide, most probably a polyamino-sugar, but it does not contain an appreciable number of acidic groups. The fact that the appearance of this compound corresponds to the disappearance of the previously described polysaccharide and the similar solubility characteristics of the two hint at the possibility that compound number 2 is a derivative of compound 1. The fact that positive Marchi staining can be obtained in the rat only 6–8 days after the onset of degeneration might be due to a slow change, probably decarboxylation, of the product (which is split off at an earlier period) into the Marchi product.

This study does not include the well known *stage* 3, during which sudanophilic material appears in gitter cells, Schwann cells and macrophages respectively.

The presence of polysaccharides other than glycogen in the nervous system has recently been studied by Swedish observers. In the calf, BRANTE (1955) found hexosamine-containing metachromatic and non-metachromatic substances in the water- and liquid-insoluble residue of normal grey and white matter. SVENNERHOLM (1956) found a compound in the brain tissue, which is not lipid-bound and which contains an amino-sugar and sialic acid.

The presence of acidic compounds in the myelin sheath has been reported by a number of observers by the use of metachromasia (FEYRTER, 1936; WISLOCKI and SINGER, 1950; LANDSMEER, 1951; WOLMAN, 1956*b*). WISLOCKI and SINGER (1950)

found that the metachromasia was due to the myelin lipids, while LANDSMEER (1951) adduced evidence that the metachromasia was due to phosphoric acid radicals. It appears obvious that the observed metachromasia was due to phospholipids as in both studies frozen sections were used. WISLOCKI and SINGER (1950) have not observed metachromasia in the neurokeratin (in paraffin sections) probably because they used a staining solution at a pH so low that the weak acid did not ionize.

The finding of a polysaccharide constituent in the myelin sheath raises the question of its relationship to the so-called neurokeratin. According to most workers in this field (STOECKENIUS and ZEIGER, 1956) modern work confirms the views of BOVERI, KÖLLIKER, RANVIER and CAJAL who regarded neurokeratin as an artefact. The formation of neurokeratin is considered to be due to the effect of fixatives which separate the lipids from the non-lipid constituents and through this 'Entmischung' or precipitation appears the network of neurokeratin. SCHÜMMELFELDER (1950) could observe the formation of this network by vital staining only in nerves which had been damaged by mechanical compression. Whereas the neurokeratin of histologists (SMITH, 1936) includes all the stainable elements of the myelin sheath which are not extracted by the lipid solvents commonly used in the preparation of histological sections, biochemists (EWALD and KÜHNE, 1874; BLOCK, 1931; LE BARON and FOLCH, 1956) consider neurokeratin as a definite protein substance. In order to avoid confusion it seems advisable to accept the biochemical definition of neurokeratin and to adopt other names for the two compounds described in this article. The name myelosaccharide A is proposed for the acidic myelin component and myelosaccharide B for the non-acidic Marchi compound.

In this study the relationship between the myelin sheath and the ground substance has not been investigated. It has been shown that besides cells and processes nervous tissue also contains a ground substance which is partly made up by carbohydrate (as mucopolysaccharide or mucoprotein; HESS, 1953), and protein (DIXON, 1956). It has also been shown (WOLMAN, 1956*c*) that the ground substance contains strongly acidic groups (probably in its polysaccharidic constituent) and thereby differs from myelin in sections stained with the Bi-Col method. Although tinctorial changes have been observed in the ground substance of degenerating nerve and brain tissue in the earliest phase of degeneration (some of the changes can be seen in the illustrations), these observations are considered to be beyond the scope of this study.

SUMMARY

Study of the degenerating myelin of rat sciatic nerve and brain by new histochemical methods revealed the following:

As from the first day of degeneration, the myelin sheath contains an increased amount of a reactive, weakly-acidic polyacid. There is evidence to suggest that this compound is a hexosamine-containing polysacharide. The amount of the compound increases during the first four days and decreases thereafter.

As from the sixth to the eighth day of degeneration, the myelin sheath contains a hexosamine-containing, non-acidic polysaccharide. It is presumed that this (Marchi-positive) compound is a derivative of the previously mentioned polysaccharide. The amount of the Marchi-compound is maximal around the twelfth day and decreases thereafter.

The relationship of these compounds to 'neurokeratin' is discussed.

REFERENCES

BLOCK R. J. (1931) *J. biol. Chem.* **94,** 647.
BRANTE G. (1955) *Biochemistry of the developing nervous system* (Edited by H. WAELSCH) Academic Press, New York.
DIXON K. C. (1956) *J. Path. Bact.* **71,** 37.
EWALD A. and KÜHNE W. (1874) cited by BLOCK (1931).
FEYRTER F. (1936) *Virchows Arch.* **296,** 645.
FEYRTER F. (1955) *Zbl. allg. Path. path. Anat.* **93,** 442.
HESS A. (1953) *J. comp. Neurol.* **98,** 69.
LANDSMEER J. M. F. (1951) *Acta physiol. pharmacol. néerl.* **2,** 112.
LEBARON F. H. and FOLCH J. (1956) *J. Neurochem.* **1,** 101.
SCHÜMMELFELDER N. (1950) *Virchows Arch.* **319,** 294.
SMITH M. C., STRICH S. J. and SHARP P. (1956) *J. Neurol. Psychiat.* **19,** 62.
SMITH P. E. (1936) *Bailey's Textbook of Histology* Wood, Baltimore.
STOECKENIUS W. and ZEIGER K. (1956) *Ergebn. Anat. EntwGesch.* **35,** 420.
SVENNERHOLM L. (1956) *Acta chem. scand.* **10,** 694.
SWANK R. L. and DAVENPORT H. A. (1934) *Stain Tech.* **9,** 11.
UZMAN L. L. (1955) *Arch. Path. (Lab. Med.)* **60,** 308.
WISLOCKI G. and SINGER M. (1950) *J. comp. Neurol.* **92,** 71.
WOLMAN M. (1956*a*) *J. Histochem.* **4,** 195.
WOLMAN M. (1956*b*) *Neurology* **6,** 636.
WOLMAN M. (1956*c*) *Bull. Res. Council Israel* **6E,** 27.
WOLMAN M. (1957) *Exp. Cell Res.* **12,** 231.

VOL.
1
1956/5

VOL.
1
56/57